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An integrated approach applied to anticancer drugs across aquatic compartments

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ABSTRACT

Anticancer drugs represent emerging contaminants in the water cycle, raising concerns due to their intrinsic biological activity, low concentrations, persistence, and potential toxicological effects. Despite growing international concern, few studies have examined their environmental fate in water resources of arid and semi-arid zones, notably in North Africa (Oujda, Morocco). This study investigated the selection rationale, occurrence, and spatial distribution of for five anticancer drugs cyclophosphamide (CP), ifosfamide (IF),5-fluorouracil (5-FU), doxorubicin (DOX) and etoposide (ETO), across aquatic compartments in Oujda, while extending the analysis to the occurrence of anticancer drugs in treated wastewater treatment plant (WWTP) effluents reused for vegetable crop irrigation. In this context, multi-residue methods combining extraction by solid-phase extraction with Oasis HLB cartridges, identification and quantification by high-performance liquid chromatography electrospray ultraviolet combined with tandem mass spectrometry. Results revealed the occurrence of major anticancer drug in hospital influents and effluents, wastewater treatment plants effluents, and groundwater at concentrations ranging from 5.866 µg. L^{-1} to 428.58 µg. L^{-1}. These findings suggest incomplete removal during conventional treatment and a possible contribution of hospital effluents to groundwater contamination. They also point to a potential recirculation route linked to irrigation with treated wastewater, provide initial evidence of anticancer drugs in the water cycle of Oujda, and emphasize the need for improved source control, wastewater treatment, as well as further research on their environmental behavior.

Keywords: anticancer drugs, selection criteria, multi-residue methods, hospital oncology, wastewater, groundwater, pseudo-persistent, vegetable crops.

INTRODUCTION

In recent years, cancer has shown a worrying increase worldwide, rising from 19,976,499 cases in 2022 to an estimated 53,504,187 cases in 2027. In Morocco, the number of cancer cases was estimated at 63,609 new diagnoses in 2022, a figure expected to reach 154,884 by 2027 (Ferlay et al., 2024; Shakambari et al., 2017). To treat this disease, a variety of methods are available: surgery, external or internal radiotherapy, and anticancer drug therapy (Gouveia et al., 2023). The latter category includes cytotoxic or anticancer drugs chemotherapy: alkylating agents (e.g. Cyclophosphamide, Ifosfamide), the Antimetabolites group (e.g. 5-Fluorouracil), the cytotoxic antibiotics groups (e.g. Doxorubicin), plants alkaloids (e.g. Etoposide); hormonal therapy (e.g. Tamoxifen) and targeted therapy (e.g. Trastuzumab), is the second commonly applied treatment (Gouveia et

al., 2023). These compounds can be administered orally or intravenously or by infusion (Česen et al., 2015). They are developed to inhibit or stop cell growth or to kill cells directly. However, they tend to act indiscriminately on both cancerous and healthy cells (Sung et al., 2021). As reported by the International Agency for Research on Cancer (IARC), cyclophosphamide and etoposide are classified as carcinogenic to humans, whereas doxorubicin is considered probably or possibly carcinogenic, and agents such as ifosfamide and 5-fluorouracil have not been classified as carcinogenic. After administration, these drugs and their metabolites are largely excreted by patients into municipal wastewater in outpatient care and into hospital effluents in inpatient settings (Santos et al., 2018; Collier, 2007). These waters are discharged directly into the sewage system without treatment at the source, and eventually reaches a wastewater treatment plant (WWTP) (Ferrando-Climent et al., 2014; Orias et al., 2015; Isidori et al., 2016). Recent studies demonstrate that conventional treatments do not achieve high removal efficiencies for these anticancer drugs, as they are typically resistant to biodegradation (Česen et al., 2016; Franquet-Griell et al., 2017; Majumder et al., 2021). This increases the probability that they remain active after wastewater treatment and contaminate river waters and groundwater and drinking water (Aydın et al., 2022; Ferrando-Climent et al., 2013; López-Serna et al., 2013; Ferrando-Climent et al., 2014; Nassour et al., 2020; Gouveia et al., 2022). To the authors' knowledge, the absence of data on the presence of anticancer drugs in Morocco's water cycle constitutes a significant knowledge gap, further compounded by limited understanding of the possible contamination pathways and the efficiency of local wastewater treatment plants. This issue is particularly concerning given that these metabolites possess cytotoxicity, genotoxicity, mutagenicity, carcinogenicity, endocrine-disrupting, and/or teratogenicity properties (Mišík et al., 2019). Moreover, current knowledge regarding their ecotoxicity and genotoxicity across different aquatic compartments remains scarce (Al Qarni et al., 2016; Luo et al., 2014; Yadav et al., 2021). These compounds present various structures and physico-chemical characteristics, including high polarity and low octanol-water coefficients, even low concentrations, of the order of ng. L^{-1} to µg. L^{-1} (Azuma, 2018; Santana-Viera et al., 2020). The precise quantification of these compounds in complex matrices remains a significant analytical challenge (Xie, 2012; Česen, Kosjek, et al., 2016; Toolaram et al., 2014), underscoring the need for a multi-residue methodology (Castellano-Hinojosa et al., 2023; Kmmerer, 2010). Solid-phase extraction (SPE) paired with high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) or ultraviolet detection (HPLC-UV/MS) provides an effective strategy for compound extraction and preconcentration, as well as for the accurate detection and quantification of target anticancer drugs.

This study addressed the limited evidence on anticancer drugs in environmental matrices from Oujda, Eastern Morocco. Five compounds were prioritized using predefined epidemiological, consumption, occurrence, and ecotoxicological criteria, then investigated in oncology hospital influents and effluents, nearby groundwater wells, and wastewater treatment plant effluents by a multi-residue SPE-HPLC-ESI-UV/MS method.

MATERIALS AND METHODS

Selection criteria for anticancer drugs

The selection of anticancer drugs was based on regional consumption data and use patterns in the Oriental region during the 2019–2021 period, obtained from institutional records of the Oncology pharmacy registration office of the Hassan II regional oncology center. Oujda. Eastern Morocco. The dataset was compiled and processed using Microsoft Excel. Selection also considered the literature evidence on reported ecotoxicological, genotoxic, endocrine-disrupting, and teratogenic effects, previous detection in hospital wastewater effluents, wastewater treatment plant effluents, and river waters, as well as key physicochemical properties such as polarity, octanol–water partition coefficients, mobility, and pseudo-persistence.

The next step involves the detection and quantification of the five selected anticancer drugs: alkylating agents, including cyclophosphamide (CP) and ifofamide (IF); antimetabolites, including an antipyrimidine, a fluorinated uracil analog, 5-fluorouracil (5-FU); cytotoxic antibiotics, containing doxorubicin (DOX) and plant alkaloids containing podophyllotoxin derivatives such as etoposide (ETO), in different aquatic matrices (wastewater, groundwater).

Multi-residue methods: SPE-HPLC-ESI-UV/MS

Standards

Standards of cytostatic compounds: cyclophosphamide, ifosfamide, 5-Fluorouracil, doxorubicin, and etoposide were provided free of charge by the Oriental Oncology Hospital Center Hassan II. Oujda. Morocco (Pharmacy Department, Oriental Oncology Center). The solutions containing 1mg/ml were prepared by dissolving the compound in HPLC-grade methanol (MeOH) daily.

Reagents and materials

All solvents were of HPLC grade, and all chemicals were of analytical reagent grade. Methanol, acetonitrile, acetone (99.8%), water, ammonium formate (98.2%), Ammonium hydroxide (25%) were all obtained from VWR Chemical (Germany). Formic acid (HPLC grade) (98–100%) was acquired by Scharlau (Spain). Water HPLC was obtained by Oxford (India). Hydrochloric acid HCL (37%) was purchased from Panreac (Spain). Dipotassium phosphate (K_2HPO_4) (98–101%) was obtained by Sigma-Aldrich (Germany). Ammonium acetate was purchased from Solvapur (Morrocco), whereas potassium dihydrogen orthophosphate (KH_2PO_4) (98–100.5%) and ethylenediamine tetra acetic acid disodium salt dihydrate Na2EDTA (99–101%) were purchased from Oxford (India). HCl 0.1 N was supplied by Sigma (Switzerland), while orthophosphoric acid was sourced from Merck (France). Oasis HLB cartridges (6cc, 200 mg) were purchased by Tecnilab-HTDS (France). Cellulose acetate (0.45 µm pore size) was supplied by Merck (France), and glass fiber filters (1.2 µm pore size) was acquired from Whatman (Filtratec, France).

Sampling scheme: composite samples, grab samples

Samples were collected from the oncology hospital HASSAN II, adjacent areas, and effluents of local wastewater treatment plants.

This public oncology hospital has 26 beds and administers chemotherapy to around 50 patients daily, with anticancer drugs prepared in the medical oncology and inpatient departments. The medical oncology department administers an average of 95 chemotherapy treatments daily, while the average number of anticancer drugs prepared for the inpatient department is 20 preparations per day (Ouasrhir, Bourhaleb, et al., 2019). All treatment protocols according to the Bulletin of Moroccan Oncology (Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016); (Ouasrhir, 2016). The oncology center treats patients with various forms of cancer, including breast cancer, Sarcoma cancer, testicular cancer, acute lymphoplastic leukemia cancer, and small-cell lung cancer (Ouasrhir, 2016). In the hospital, anticancer drug management begins with their reception and storage, followed by pharmaceutical validation of therapeutic protocols. Drugs are then prepared by pharmacy technicians in a specialized room with an isolator and air filtration system, before being administered to patients. Proper handling of medical waste and expired drugs is also ensured throughout the process (Ouasrhir et al., 2019).

The influents and effluents of the oncology center are stored in two septic tanks, one for inpatient chemotherapy treatment influents and the other a general septic tank for all the effluents of the oncology center. These tanks are periodically emptied and transported to the Oujda wastewater treatment plant (WWTP). This plant has a complete treatment process, from pre-treatment to tertiary treatment, with a treatment capacity of 860,000 population equivalent for a wastewater flow of 65,000 m3/d. After treatment, the treated effluent is discharged into the Oued Bounaim (Oujda, Eastern Morocco) (Kajeiou et al., 2023).

Water samples were collected from four sources. Septic tank influents (S1) and general septic tank effluents (S2) were obtained from the Hassan II Regional Oncology Hospital in Oujda (Eastern Morocco) with permission from the hospital management (File.1-SI Supplementary administrative document). Treated wastewater effluents (S3) discharged into the Oued Bounaim in Oujda (Eastern Morocco) were sampled outside the treatment facility, and groundwater samples (S4) were collected from a well at a depth of 100-120 meters on a farm near the hospital (200 meters). The collection, storage, and transport of samples for analysis, as outlined in the developed sampling program, were performed in accordance with the Moroccan Standard NM ISO 5667–1:2012 – Water Quality: Sampling, Guidelines for the design of sampling programs and techniques. Wastewater samples were collected as 24-hour composite samples, with collection times at 8:00, 11:00, 16:00, 18:00, and 8:00 the following day. In contrast, groundwater samples were collected as spot samples. Sampling was performed in June

2023 (IF, 5-FU, DOX, ETO) and in December 2023 (CP). The choice the sampling timing were guided by the average hospital stay, to reflect periods of highest potential drug discharge into wastewater. All samples were stored in 1 L amber HDPE bottles previously rinsed with ultrapure water and were subsequently refrigerated at 4 °C for preservation.

Samples treatment

The extraction of the samples was usually carried out within 24h of collection to keep microbial degradation to a minimum and to prevent the potential degradation of target compounds (Seira, 2013; Negreira et al., 2013; Santana-Viera et al., 2019). All samples (100 ml) were centrifuged at 8.000 rpm for 10 min to eliminate suspended matter and filtered through 1.2 µm fiberglass filter followed by 0.45 µm cellulose acetate membrane filters (Yang et al., 2011; Nassour et al., 2020). The waters were preconcentrated by SPE using Oasis HLB (200 mg, 6 cc) extraction cartridge. Two analytical methods were previously developed by (Martín et al., 2014) for the fraction of neutral (CP, IF, DOX, and ETO) or acidic (5-FU) molecules (Santana-Viera et al., 2019);(Seira, 2013). To avoid cross-contamination during sample pretreatment from different stations, a 100 ml sample of water HPLC was used. This was passed through after each pretreatment process. In the first method for the fraction of neutral, the samples were acidified to pH between 2.5 and 3.5 with HCl 0.1 N, adjusted with solution of K_2HPO_4 and KH_2PO_4 for pH 6.8, then the chelating agent 100 µl Na_2EDTA 10% solution (0.01% (w/w)) was added. The cartridges were conditioned with 6 ml of methanol HPLC and 6 ml of water HPLC with 0.1 M ammonium acetate. The cartridges were loaded with 100 ml of waters and afterwards were washed with 6 ml of Water HPLC. After drying (30–45 min), 6 ml of methanol HPLC and 6 ml of formic acid/methanol (5/95) for 1 ml/min flow rate. Followed by rotary evaporation at 25 °C and recuperate to 2 ml amber glass vials containing 1 ml of methanol HPLC. The second method or the fraction of acidic, the samples were first adjusted to pH 12 and then acidified to pH 3 with orthophosphoric acid. The cartridges were conditioned with 6 ml of methanol and 6 ml of water HPLC. The cartridges were loaded with 100 ml of waters and afterwards were washed with 6 ml of Water HPLC. After drying (30–45 min), 6 ml of methanol and 6 ml of methanol/ammonium hydroxide 0.5% (95/5) (flow rate 1ml/min) were added, followed by rotary evaporation at 25 °C and recuperate to 2 ml amber glass vials containing 1 ml of methanol HPLC. All analyses were performed in duplicate (n=2). The SPE procedure steps and samples concentration (S1, S2, S3 and S4) are summarized in Figure 1.

Equipment and chromatography conditions

Chromatographic analyses were performed using an HPLC Ultimate 3000 (Dionex) coupled with UV-Visible PDA diode array detectors and Exactive Plus mass spectrometer equipped with positive and negative electrospray ionization (ESI^+, ESI^-). The mass spectrometer operated under the following optimized conditions: The curtain gas was set to 10 V, the source temperature was 700 °C, and the collision gas had a high nitrogen content. The ion spray voltage was set to 4000 V, and the ion source gases GS1 and GS2 were set to 40 V and 60 V, respectively (Thermo Scientific, USA). Separation was achieved using the BDS Hypersil C18 (150 × 4.6) mm × 5 µm analytical column (Thermo Scientific, USA). Separation of analytes was performed using a gradient elution method with two mobile phases: phase A consisted of acetonitrile/ultra-pure water (1:9, v/v), and phase B consisted of acetonitrile/ultra-pure water (9:1, v/v), both buffered with 1 mM ammonium acetate (NH_4CH_3COO) and 0.3% formic acid (HCOOH). Elution was performed at a flow rate of 0,3 ml/min with the column temperature kept at 25 °C. All data were acquired and processed using Therm Xcalibur Quan Browser. To integrate UV/Vis and analog chromatograms, the Avalon integration algorithm was used (Santana-Viera et al., 2020; Seira, 2013; Negreira et al., 2013; Santana-Viera et al., 2019; Seira et al., 2013; Negreira et al., 2014).

Quantification, method validation, and mass spectral characterization

The analytical performance of the method was examined through the assessment of linearity (R^2), sensitivity parameters (LOD and LOQ), and repeatability (expressed as SD and RSD). Linearity was tested using calibration standards prepared in HPLC-grade methanol, with concentrations ranging from 1 to 1000 µg. L^{-1}. For each pharmaceutical analyte, calibration curves were established using three to five concentration

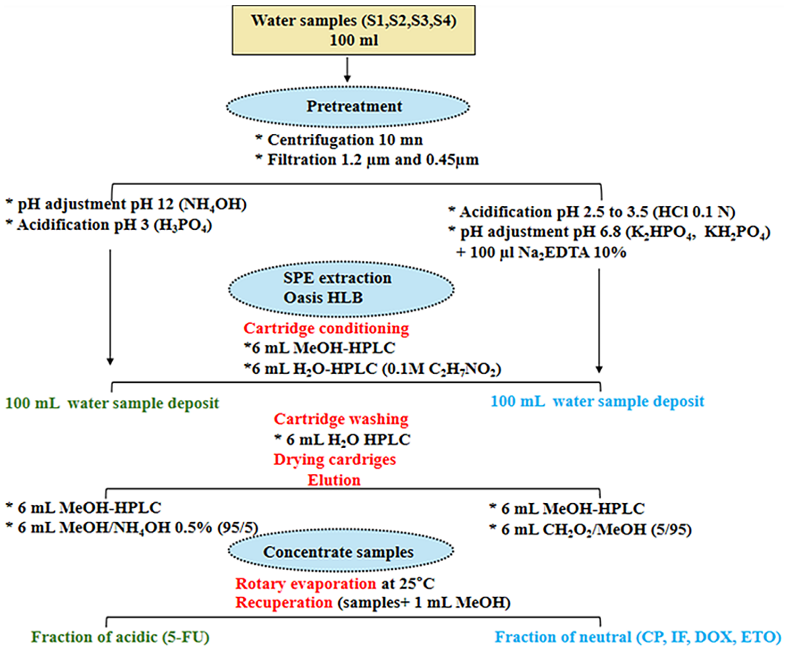

Figure 1. Schematic representation of the SPE procedure and sample concentration

levels by injecting the corresponding standard solutions. The limit of detection (LOD) and quantification (LOQ) were theoretically estimated from the calibration curve. The σ/S approach was used, based on linear regression analysis performed in Excel. In this method, LOD and LOQ are calculated using the slope of the calibration curve (S) and the standard deviation of the residuals (σ). According to the ICH guidelines for analytical method validation, LOD is defined as the lowest concentration that produces a signal-to-noise ratio > 3, whereas LOQ is represents as the lowest concentration that produces a signal-to-noise ratio > 10 (Martín et al., 2014).

RSD (relative standard deviation) quantifies the dispersion of results and evaluates the repeatability of measurements.

Internal standards are generally used to assess matrix effects (EM). However, in the present study, matrix effects were not evaluated, and the high cost of isotopically labeled internal standards further limits their routine application (Luo et al., 2014). To overcome this limitation and ensure reliable quantification, the solid-phase extraction (SPE) procedure, chromatographic separation, and electrospray ionization (ESI) parameters were optimized, reducing matrix related interferences and improving analytical accuracy (Cortese et al., 2020; Besse et al., 2012).

Quantification of the drugs was performed using multiple reaction monitoring (MRM), selecting the two most specific precursor-to-product ion transitions for each analyte. Concentrations were determined by comparing the peak areas of the analytes with those of their corresponding external standards using calibration curves. Compound identity was confirmed by aligning retention times and MRM transitions with authentic standards. The drugs analyzed in positive ion mode (ESI^+) were quantified using CP, IF and ETO, while 5-FU and DOX was also analyzed in negative ion mode (ESI^-). To prevent cross-contamination, HPLC-grade methanol was injected between samples, followed by a rigorous column cleaning protocol using acidified ultrapure water (pH 3) and HPLC-grade acetonitrile (ACN).

To ensure unequivocal identification of all target compounds and to minimize the risk of false positives, a set of confirmation criteria was established. In accordance with Directive 2002/657/EC, these criteria comprised: a retention-time deviation of less than 2% between analytical standards and the corresponding samples; and accurate-mass measurements for precursor and fragment ions with a maximum tolerance of ±5 ppm. Moreover, only the samples exhibiting an isotopic pattern agreement exceeding 90% were considered as positively identified (Gómez-Canela et al., 2012). For each investigated compound, the results were expressed as the maximum measured environmental concentration (MECmax) recorded in the studied aquatic matrices.

RESULTS AND DISCUSSION

Results

Selected anticancer drugs established based on consumption patterns, physicochemical properties, and environmental fate

The selection of target anticancer drugs was based on several criteria. Firstly, epidemiological studies conducted between 2019 and 2021 were evaluated on primary breast cancer (43%) secondly lung cancer (12%), thirdly colon cancer (10%), stomach cancer, cervical cancer and prostate cancer (8%,7%, 7%) respectively, in fourth position rectal cancer (5%) and ovary, rectum (4%) (see Figure 2) [Regional Hassan II Oncology Center. Oujda. Eastern Morocco. These results similar to Maamri et al. (Abdelatif, 2015).

According to the epidemiological study conducted between 2019-2021, certain antineoplastic drugs used in the hospital were identified as the most frequently administered, notably 5-FU, CP, and IF exhibited comparatively higher consumption levels (average 81.697 g/month, 32.138 g/month, 28.861 g/month, respectively) than ETO and DOX (3.167 g/month, 7.962 g/month). Since these drugs are primarily administered to hospitalized patients, they were considered relevant for selection as target compounds due to their relatively higher likelihood of excretion into hospital influents and effluents [Oncology pharmacy registration office of the Hassan II regional oncology center. Oujda. Eastern Morocco].

As shown in Table 1, the literature review identified several selected anticancer drugs as compounds of potential environmental concern based on previously reported ecotoxicological, genotoxic, endocrine-disrupting, and teratogenic effects. This compilation of published evidence further supported their prioritization for occurrence analysis in the studied aquatic compartments**.**

In this study, these substances display extended environmental half-lives, varying between 38 days for CP and 60 days for 5-FU, and up to 180 days for IF, ETO, and DOX, while sediment half-lives spanned 338 for CP to 1620 days for DOX and ETO (Castellano-Hinojosa et al., 2023). Among the studied drugs, most antineoplastic drugs are highly soluble with predicted log Kow values ranged from –0.89 5-FU to 1.27 DOX, and mobile (HSDB, 2019.Hazardous Substances Data Bank, 2019); (*Search and Share Chemistry*, 2019; Mahoney et al., 2003; Weylandt et al., 2007; de Oliveira Klein et al., 2021).

International studies document the widespread environmental occurrence of five principal anticancer drugs across aqueous matrices, with concentrations spanning from trace to exceptionally high levels. Cyclophosphamide and ifosfamide reach peak levels in hospital effluents (>680,000 ng. L^{-1}) (Hamon et al., 2018). 5-Fluorouracil (5-FU) is detected from <0.5 to 124,000 ng. L^{-1} in hospital effluents (Isidori et al., 2016), in WWTP and persists in river waters (Gómez-Canela et al., 2012). The environmental occurrence of doxorubicin is confirmed in wastewater treatment plants at up to 10,400 ng. L^{-1} (Souza et al., 2018), while etoposide is found in both wastewater (≤110 ng. L^{-1}) and activated sludge (≤43,000 ng. kg^{-1}) (Aydın et al., 2022). Cyclophosphamide is also reported in surface waters, demonstrating its persistence in the aquatic environment at levels up to 1.9 µg. L^{-1}. Removal

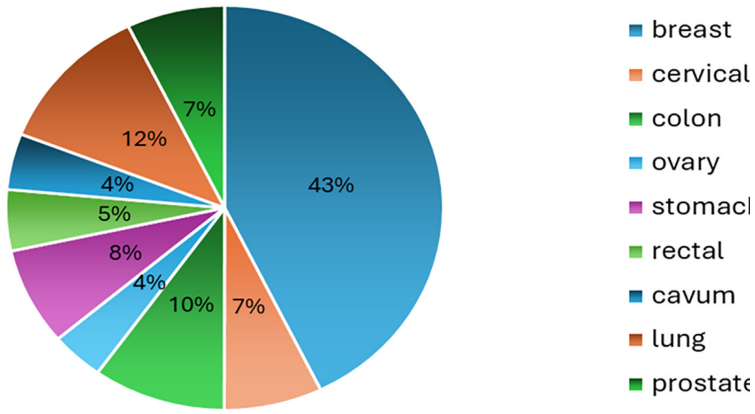

Figure 2. The epidemiological cancer study between (2019-2021) in Hassan II Oncology Hospital

Table 1. Environmental toxicological risks in aquatic systems

Anticancer drugs	Toxicity to aquatic organisms	References
CP	Genotoxic, endocrine-disrupting and/or teratogenic	(De and Lutte, 2019), (Novak et al., 2017); (Česen et al., 2016);(Gajski et al., 2018) ; (Russo et al., 2018)
IF	Genotoxic, endocrine-disrupting and/or teratogenic	(De and Lutte, 2019), (Russo et al., 2018); (Gajski et al., 2018) ;(Novak et al., 2017)
5-FU	Concentration-dependent toxicity, genotoxic, endocrine-disrupting and/or teratogenic	(Załeska-Radziwiłł et al., 2014); (Parrella et al., 2014); (Russo et al., 2018); (Zounkova et al., 2010); (De and Lutte, 2019)
DOX	Low toxicity (few studies), High genotoxic	(De and Lutte, 2019); (Załeska-Radziwiłł et al., 2014);(Zaleska-Radziwill et al., 2011); (Parrella et al., 2015);(Zounkova et al., 2010)
ETO	Toxic, genotoxic, endocrine-disrupting and/or teratogenic	(De and Lutte, 2019); (Russo et al., 2018); (Parrella et al., 2014)

rates for the investigated compounds have been reported to vary widely, ranging from 0% to 100%, depending on the specific properties of each anticancer agent and the treatment technologies applied, as shown in several studies (Usawanuwat et al., 2014; Calza et al., 2014; Lutterbeck et al., 2020; Borzyszkowska et al., 2016). To the best of our knowledge, available data on the occurrence of anticancer drugs in Morocco remains scarce.

Multi-residues approach: SPE-HPLC-UV/MS

Oasis HLB cartridges were employed for SPE, offering effective retention of analytes like CP, IF, 5-FU, DOX, and ETO, owing to their sensitivity to differences in polarity, pH, and physicochemical characteristics. The three variables considered were sample volume (100 mL), pH adjustment, and acidification (pH 3, 6.8, and 12, adjusted using NH_4OH, H_3PO_4, and HCl) (see Figure 1) (Santana-Viera et al., 2019; Seira, 2013).

Method validation

Quality parameters of the developed method: Coefficients of determination ($R^2 > 0.9985$) were obtained for the majority of the compounds in the concentration range from 1µg. L^{-1} to 1000 µg. L^{-1}. LOD and LOQ values ranged from 0.001 to 46.60 ng. $L^{-1}$and from 0.0033 to 141.22 ng. L^{-1}, respectively. The LOD, LOQ, average, SD and RSD (%) values are presented in Table 2.

Method quantification (ion chromatograms) and mass spectral characterization

The extracted ion chromatograms of cyclophosphamide, ifosfamide, 5-fluorouracil, doxorubicin and etoposide are shown in Figure 3.

Standard solution containing: (a) 100 µg. L^{-1} of CP, (b) 500 µg.L^{-1} of IF, (c) 500 µg.L^{-1} of 5-FU, (d) 100 µg.L^{-1} of DOX, (e) 100 µg.L^{-1} of ETO. Anticancer drugs detected in matrices aquatic: (f) CP in effluents samples of a wastewater treatments plan EWWTP; IF, (g) 5-FU and DOX in effluents hospital wastewater EFHWW and (h) in groundwater GW.

The main MS/MS results obtained for the investigated compounds are summarized in the table below, including precursor and product ions, ionization modes, and comparison with values reported in previous studies. These transitions confirm the molecular identities and are consistent with those found in the literature, further supporting the reliability of the analytical method used (see Table 3).

Table 2. Quality control parameters of the HPLC-UV/MS method in methanol: retention time (RT), linearity (R^2), LOD, LOQ, Average, SD, and repeatability (RSD)

Drugs	RT (min)	Linearity (µg. L^{-1})	Regression equation	R^2	LOD (ng. L^{-1})	LOQ (ng. L^{-1})	Average	SD	RSD %
CP	21.45	1–500	Y=241003+216.95X	0.99	46.60	141.22	270058	3085.81	1.14
IF	14.36	5–500	Y=84433+145.9X	0.99	21.39	64.83	110841	945.87	0.85
5-FU	3.42	1–1000	Y=- 916613 +41791X	0.99	0.001	0.0033	9029645	16030347	0.0001
DOX	10.67	1–500	Y=-174313+ 37174.7X	0.99	18.361	55.64	4769725	184996.25	4.33
ETP	10.46	1–100	Y=55058.4+22635.7X	0.99	11.84	35.90	3265823	81266.86	2.48

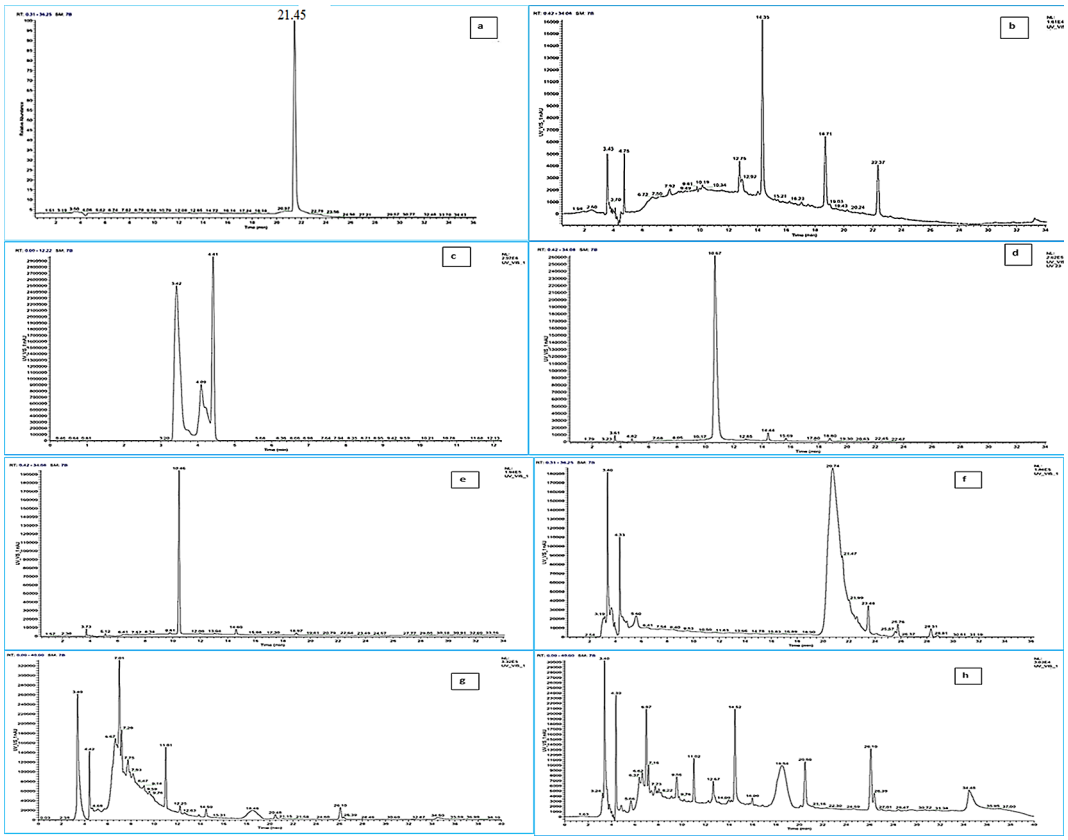

Figure 3. Ion chromatogram of a standard solution and an anticancer drug in different matrices aquatic in Jun (IF, 5-FU, DOX, TO) and in December for CP

Table 3. Molecular and product ion characteristics of target compounds

Compound	Retention time (min)	Ionization mode	Molecular ion (m/z)	Product ion (m/z)	Litterature support
CP	21.45	ESI^+	261.0	140.0	(Santana-Viera et al., 2019)
IF	14.36	ESI^+	261.0	154.0	(Santana-Viera et al., 2019)
5-FU	3.42	ESI^-	129.0	85.92	(Search and Share Chemistry, 2019), (Mahoney et al., 2003), (Mullot et al., 2010),(Seira et al., 2013)
DOX	10.67	ESI^-	542.2	395.1	(Search and Share Chemistry, 2019),(Mahoney et al., 2003),(Mullot et al., 2010),(Seira et al., 2013)
ETO	10.46	ESI^+	587.2	185.0	(Santana-Viera et al., 2019)

Determination of anticancer drugs levels in wastewater and groundwater

The occurrence data are presented as MEC-max values for the investigated anticancer drugs (CP, IF, 5-FU, DOX and ETO) in various types of wastewater (influent hospital wastewater (IF-HWW), effluent hospital wastewater (EFHWW), effluent wastewater treatment plan (EWWTP) discharging into Oued Bounaim, and groundwater (GW) grab samples collected during June and December 2023 as described in the experimental section. The results are presented in Figure 4.

Discussion

The framework used in this study offered an initial basis for prioritizing anticancer drugs by combining local epidemiological information, regional consumption data, physicochemical properties, and available toxicological evidence. A key strength of this approach is that it reflects local conditions, rather than relying solely on generalized prioritization schemes (Kümmerer et al., 2016). The subsequent detection of selected compounds in hospital effluents, WWTP effluents, and surface waters gave additional relevance to this

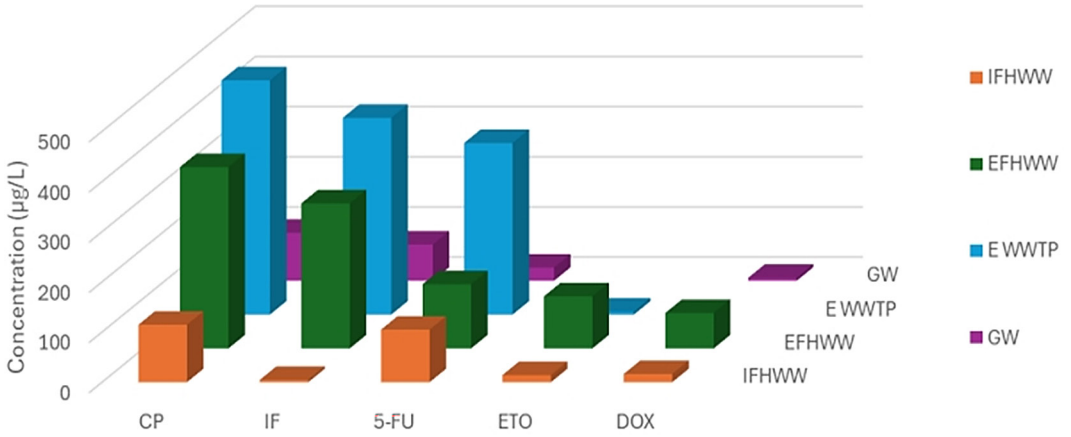

Figure 4. Occurrence of anticancer drugs in wastewater and groundwater samples (MEC_{max};µg.L^{-1})

prioritization. However, the approach remains indirect, since actual emissions are influenced by excretion profiles, hospital practices, and temporal variation in drug use. Toxicological data reported for several compounds, including mutagenic, teratogenic, and fetotoxic effects, support their consideration as compounds of concern (Booker et al., 2014), but do not allow their occurrence in aquatic environments to be inferred. The currently available ecotoxicological evidence is also too limited to allow a reliable transposition of human toxicity data to environmentally relevant chronic exposure conditions (Castellano-Hinojosa et al., 2023). Similar limitations apply to physicochemical descriptors. Kow, Koc, and half-life values are informative, but they do not fully capture the behavior of polar and ionizable compounds and remain strongly dependent on site-specific conditions (Kümmerer et al., 2016; Azuma, 2018; Harrison et al., 2025). The occurrence of these compounds in WWTP effluents and surface waters is consistent with incomplete removal by conventional treatment, although this interpretation must remain cautious given the variability in treatment performance and compound-specific behavior. Taken together, these limitations indicate that the framework is better viewed as a preliminary prioritization tool that gains value when interpreted alongside measured concentrations in the different aquatic matrices investigated.

Multi-residue methods: SPE-HPLC-ESI-UV/MS

Solid-phase extraction (SPE) was carried out using an Oasis HLB cartridge, chosen for its balanced hydrophilic–lipophilic characteristics that improve sensitivity for highly polar analytes. The three variables considered were the sample volume, pH adjustment, and acidification of the wastewater (100 mL) to prevent cartridge clogging, a precaution applied even for groundwater samples (Santana-Viera et al., 2019; Negreira et al., 2013).

For the acid fraction, the pH is adjusted to 12 (NH_4OH) to deprotonate 5-FU (very polar) and promote anionic interactions with quaternary ammonium via Coulomb forces (electrostatic), then acidified to pH = 3.5, and during the elution phase using a basic organic solvent MeOH (NH_4OH) intended to protonate the molecule and facilitate its recovery. Studies have obtained identical results for 5-FU (Seira, 2013; Vaudreuil et al., 2020).

For the neutral fraction, the sample pH is acidified to 2 to protonate CP, IF, DOX, ETO, and promote electrostatic cationic interactions with chloridic acid, then adjusted to pH 6.8, then during elution, unlike 5-FU, by adding a more acidic organic solvent (MeOH (HCOOH)) to deprotonate and easily recover CP, IF, DOX, ETO (Seira, 2013). The compounds display chemically diverse profiles, with pKa and log Kow values ranging from 1.54 to 9.8 and –0.89 to 1.27, respectively. Such variability requires careful optimization of extraction conditions. Accordingly, Oasis HLB cartridges were employed for both wastewater and groundwater samples. Previous studies have confirmed that Oasis HLB cartridges provide efficient SPE of acidic, basic, and neutral pharmaceutical compounds, resulting in high extraction yields and recovery percentages (Mahnik et al., 2007; Santana-Viera et al., 2019; Seira, 2013; Ferrando-Climent et al., 2014).

For most target compounds, the method provided excellent linear responses, with (R^2> 0.9985) over concentrations from 1 µg.L^{-1} to 1000 µg.L^{-1}. The LOD and LOQ values range from 0.001 ng.

L^{-1} to 46.60 ng.L^{-1} and from 0.0033 ng.$L^{-1}$to 141.22 ng.L^{-1}, respectively, and exhibit significant variation. CP has the highest level of detection (LOD). This may be due to its poor fragmentation by electrospray, resulting in low sensitivity.

The calculated limits of detection (LOD) and limits of quantification (LOQ) varied considerably due to the differences between the compounds detected and the aquatic matrices studied. These results were within an acceptable range for the concentrations in which the compounds were found (Santana-Viera et al., 2019; Negreira et al., 2013). The results obtained showed good relative standard deviation (RSD) below 15% (Negreira et al., 2013).

The mass spectra obtained via electrospray ionization in both positive (ESI^+) and negative (ESI^-) modes closely matched literature data, supporting the reliability of compound identification under the analytical conditions used. This alignment highlights the robustness and reproducibility of the method, even when applied to structurally diverse compounds and complex sample matrices (Search and Share Chemistry, 2019; Mahoney et al., 2003; (=Mullot et al., 2010; Seira et al., 2013).

To overcome the methodological limitation related to the direct quantification of matrix effects (MEs), several optimizations have been implemented to indirectly reduce their impact. Sample purification by SPE effectively reduced interferences prior to injection (Matuszewski, 2006; Raterink et al., 2014; Moein et al., 2017). Additionally, optimized HPLC separation, particularly through gradient and pH adjustments, minimized co-elutions, resulting in chromatographic profiles similar between standards and various aquatic matrices (see Figure 4) (Weitzel et al., 2011; Chambers et al., 2007; Marchi et al., 2010). The mass spectrometry detection conditions were also adjusted. Garcia-Ac et al., evaluated various ionization techniques and reported that ESI provides enhanced sensitivity and selectivity (Garcia et al., 2011). The ESI^- ionization mode, depending on the nature of the molecule, was preferred as it generally generates fewer interferences than the ESI^+ mode (Antignac et al., 2005). Furthermore, the low limits of quantification (LOQ) obtained (see Table 2) reflect the effectiveness of background noise subtraction (Gachet et al., 2015; Lai et al., 2015).

Incidence of anticancer drugs in wastewater and groundwater. Presence of anticancer drugs in hospital and wastewater treatment plant samples

Maximum reported concentrations were considered to reflect the upper range of values reported in the literature for compound prioritization. All five pharmaceuticals were detected in hospital wastewater influent (S1) and effluent (S2). Overall, MECmax values were higher in effluents than in influents, ranging from 4.592 to 467.036 µg L^{-1} depending on the compound (see Figure 4). Elevated drug concentrations in the general septic tank (S2), which collects all hospital effluents, can be attributed to a combination of institutional and pharmacological factors. This increased pharmaceutical load mainly reflects the use of anticancer drugs at therapeutic doses within the oncology hospital, in line with standard clinical protocols (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*); (Ouasrhir, 2016). Contributing factors include the pharmacokinetic and pharmacodynamic properties of each compound (biological half-life and excretion rates), exclusive intravenous administration, and the number of inpatients and outpatients (Gouveia et al., 2023). Hospitalization periods, depending on chemotherapy protocols and cancer types (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*); (Ouasrhir, 2016; Kümmerer et al., 2016; Nassour et al., 2020), as well as the environmental stability of these substances in wastewater (Ferrando-Climent et al., 2013; Nassour et al., 2020), further sustain their persistence in the collection system. For cyclophosphamide, MECmax levels were greater at 361.54 µg.L^{-1} in S2 than at 114.67 µg.L^{-1} S1, indicating clear differences between the sampling sites (Gouveia et al., 2023), and surpassing values found in the literature (Česen, Eleršek, et al., 2016; Gómez-Canela et al., 2012; de Oliveira Klein et al., 2021). Hamon et al. (2018) showed the highest levels of CP in French hospital wastewater with maximum values 687 µg.L^{-1}, well above the levels recorded in the present study. Thus, studies investigating the occurrence of CP in hospital wastewater consistently demonstrate marked differences in concentration. Cyclophosphamide, used to treat various cancers, including lymphomas, leukemia, ovarian, and breast tumors (Emadi et al., 2009; Lutterbeck et al., 2020), is administered in therapeutic doses of

700–1200 mg, and patients are treated outpatient and /or inpatients 1–2 days (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016), with hospital average consumption around 32.138 g/month. Its short biological half-life (3–12 h), partial excretion unchanged in urine (10–20%) (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016), chemical stability, and high water solubility all contribute to the elevated concentrations detected in hospital wastewater (HSDB, 2019. Hazardous Substances Data Bank, 2019; *Search and Share Chemistry*, 2019). It should be noted that CP was classed by the IARC as carcinogenic to humans (Gouveia et al., 2023). For ifosfamide, was found of MEC-max concentrations 289.13 µg.L^{-1} in S2, these result is generally consistent with previous reports, albeit slightly higher (Isidori et al., 2016). This compound is routinely monitored in hospital effluents (Gouveia et al., 2023), with peak concentrations identified in France by the same team who reported on cyclophosphamide 682.0 µg.L^{-1} (Hamon et al., 2018), exceeded those detected in the present work. The relatively high concentration of ifosfamide observed in hospital effluents samples may be explained by its high therapeutic administration at doses of 2000–5000 mg and a monthly hospital usage of approximately (average 28.861 g/month), with hospitalization lasting 3–5 days, together with its low biological half-life (4–8 h) and partial urinary excretion of the unchanged compound (10–20%) (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016), contribute to its continuous discharge. Moreover, the compound's chemical stability and pronounced water solubility further support its persistence in wastewater systems (HSDB, 2019.Hazardous Substances Data Bank, 2019; *Search and Share Chemistry*, 2019). For 5-fluorouracil, it detected the MEC-max concentration of 127.93 µg.L^{-1} in S2 relatively higher than 104.893 µg.L^{-1} in S1, which are in accordance with previous reports (Lin et al., 2014); (Luo et al., 2014); (Weylandt et al., 2007). Despite being high consumption records (average 81.697 g/month) more frequently than CP, IF, this compound exhibits a very short biological half-life of about 14 minutes (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016), and patients are generally treated as outpatients (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016). These factors likely result in concentrations lower than those of CP and IF in hospital wastewater. However, its high water solubility and chemical stability contribute to its persistence in the effluents (HSDB, 2019.Hazardous Substances Data Bank, 2019), (*Search and Share Chemistry*, 2019). Following 5-fluorouracil, etoposide was detected at moderate levels in hospital wastewater, with concentrations 103.752 µg. L^{-1} in the effluents (S2) being relatively higher than those in the influents (S1) 14.222 µg. L^{-1}**.** These concentrations observed in this study align with recent reports (Mondal et al., 2019; Olalla et al., 2018; Ferrando-Climent et al., 2014). These findings may be explained by the etoposide has low average consumption records **(**3.167 g/month), and its administered low therapeutic doses (30–150 mg·L^{-1}) (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016). Approximately 56% and 44% of ETO are excreted via urine and feces, respectively, with excretion potentially occurring over up to 120 hours, consistent with patient hospitalization periods of 3–5 days (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco, 2016*; Ouasrhir, 2016).

For doxorubicin, the mean concentrations observed in the effluents (S2) (70.589 µg.L^{-1}) exceeded those in the influents (S1) (16.455 µg.L^{-1}), aligning with the values reported in earlier studies (Souza et al., 2018; Mahnik et al., 2007). This can be attributed to the relatively low hospital consumption of this compound (7.962 g/month), together with its administration at therapeutic doses ranging between 100 to 260 mg·L^{-1}. Only a small proportion is excreted unchanged in urine (approximately 5–12% within five days) (Gouveia et al., 2022). This limited and slow excretion, together with its strong affinity for adsorption onto particulate matter (Wu et al., 2026), may account for its low detection frequency in hospital wastewater, even lower than that of etoposide. The International Agency for Research on Cancer (IARC) has classified doxorubicin as a probable human carcinogen (Group 2A), supported by sufficient evidence in animals and limited evidence in humans (Gouveia et al., 2022). The observed concentrations of anticancer drugs in hospital effluents reflect the administered dose, treatment duration, elimination pathways, and pharmacokinetic and pharmacodynamic properties, as reported in the literature (Gouveia et al., 2023). Individual patient variability and active metabolite formation may further influence these

levels. These concerning concentrations underscore the need for environmental risk assessment and for hospital authorities to consider appropriate management actions.

In the wastewater treatment plant effluent discharged into Oued Bounaim, CP, IF, and 5-FU were found at comparatively high MEC-max concentrations (467.036 µg. L^{-1}, 392.274 µg. L^{-1}, 342.067 µg. L^{-1} respectively) in the effluent from the oncology center. These results are consistent with the study by Negreira et al. (2014). Chemotherapy protocols typically span durations between 15 min to 8 hours, meaning that CP and bolus administrations of 5-FU prescribed in outpatient chemotherapy would be likely excreted at home by ambulatory patients. Beyond this pathway, CP and IF were also prescribed in internal medicine and pediatric departments outside the oncology center of the Mohammed VI University Hospital Complex (*Guide Des Protocoles Thérapeutiques En Oncologie, 3 Eme-Ver. Morrocco,* 2016; Ouasrhir, 2016), the effluents of which are discharged into the WWTP. Inter-patient pharmacokinetic variability at the time of sampling may also have influenced excretion patterns (Česen et al., 2016). These factors could therefore have contributed, at least partly, to the elevated concentrations measured in WWTP effluents.These compounds CP, 5-FU, and IF would also be resistant to biodegradation at high concentrations (Souza et al., 2018; Steger-Hartmann et al., 1997; Asad et al., 2012; Yang et al., 2011). Following Negria et al. (2014), CP and IF were among the most stable of the 26 cytostatic drugs and metabolites tested in wastewater, remaining intact for over a month at 25 °C. Although adsorption can contribute to the removal of certain compounds during treatment, it is unlikely to significantly affect CP, IF, or 5-FU due to their low octanol–water (log Kow < 1) (HSDB, 2019. Hazardous Substances Data Bank, 2019; Negreira et al., 2014) and organic carbon–water (Koc < 100 $L \cdot kg^{-1}$) partition coefficients. This promotes their mobility, persistence, and transport into surface and groundwater, while limiting their adsorption to sediments (Fonseca et al., 2017; Ghafuria et al., 2018). Consequently, their removal through adsorption in wastewater treatment plant sludge is limited, which could thus elevate the risk of environmental release (HSDB, 2019.Hazardous Substances Data Bank, 2019; *Search and Share Chemistry*, 2019). For ETO was measured at levels up to 5.866 µg. L^{-1}, lower than in the effluents from oncology hospitals. Other studies have reported low concentrations of etoposide in wastewater treatment plant effluents (Isidori et al., 2016; Castellano-Hinojosa et al., 2023). Castellano-Hinojosa et al. recently detected etoposide in activated sludge at concentrations ranging from <1 to 43 $\mu g \cdot kg^{-1}$ (Castellano-Hinojosa et al., 2023). The compound can adsorb onto suspended solids in aquatic systems, where it resists degradation and may accumulate in sediments and soils (Azuma, 2018). This behavior may result from the rapid reaction of etoposide with free chlorine used in wastewater disinfection (Negreira et al., 2014). Importantly, etoposide is classified by IARC as carcinogenic to humans (Group 1), and its presence in wastewater represents a potential risk to human health and environmental safety (Gouveia et al., 2022). DOX was not detected or was below the detection limit in the WWTP effluent discharged to the surface water of the Oued Bounaim. These results are close to other studies conducted by Negreira et al. (2014), and Isidori et al., (2016). This could be due to the transformation of the molecule into metabolites in cationic form at pH 5 to 9, which could bind it to suspended solids. In addition, its high molecular weight may contribute to its accumulation in sediments (Évaluation Préalable Finale Pour Le Défi Concernant Le Numéro de Registre Du Chemical Abstracts Service Environnement Canada, 2008). It may also be susceptible to direct photolysis by sunlight (Negreira et al., 2014; Yin et al., 2010) explained that the DOX molecule can react with the free chlorine used as a disinfectant in wastewater treatment plants, resulting in its partial or complete transformation and thus concentrations below the detection limit of the analytical methods used. The cyclic structure of DOX remains stable after primary biodegradation, as shown in studies by Mahnik et al. (2007) (see Figure 5). Based on the results obtained, CP, IF, 5-FU, and ETO are considered pseudo-resistant "contaminants" (Gouveia et al., 2023; Santana-Viera et al., 2019; Castellano-Hinojosa et al., 2023). These substances appear to be poorly eliminated by conventional secondary treatment in wastewater treatment plants (WWTPs), resulting in continuous and regular discharges to the aquatic environment. This poses a potential risk due to their persistence and bioaccumulation in various types of aquatic organisms (Santana-Viera et al., 2019; Ebele et al., 2017). The concentration of CP, IF,5-FU was detected in the receiving environment may reach levels of concern. ETO is present at low concentrations in water, they may

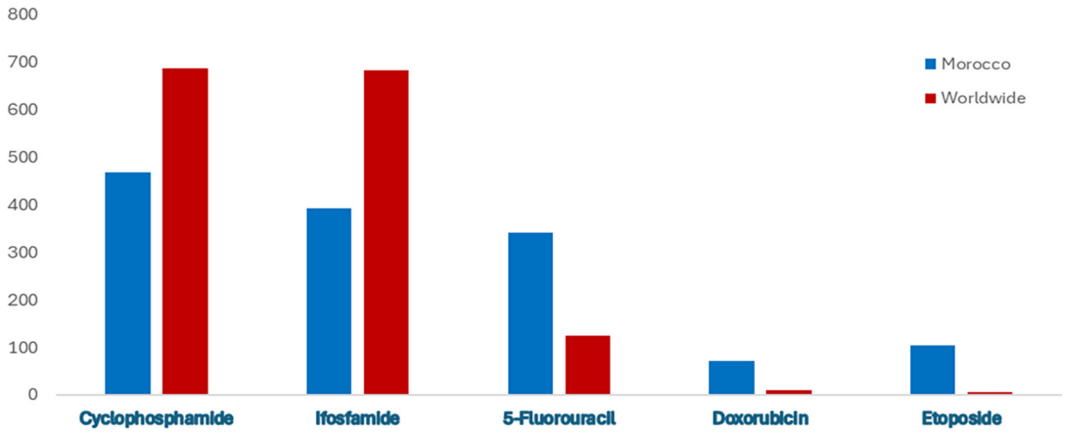

Figure 5. Comparison of anticancer drug concentrations MEC_{max} (µg.L^{-1}) observed in this study with literature data

reflect adsorption onto suspended solids or accumulation in activated sludge poses a heightened carcinogenic risk (Azuma, 2018; Gouveia et al., 2023). Conversely, doxorubicin DOX appears to be predominantly accumulated in sediments (Azuma, 2018). It is also noteworthy that the effluent discharged from the wastewater treatment plant (WWTP) into Oued Bounaim belongs to category B, which authorizes its reuse for the irrigation of cereal, industrial and forage crops, as well as pastures and tree plantations (Kajeiou et al., 2023). This suggests a potential contamination loop associated with wastewater reuse, in which anticancer residues may be redistributed within agricultural environments through transfer to soils and crops, with possible implications for food-chain entry. Although this pathway remains to be confirmed, it may represent a source of indirect human exposure and a matter of concern for surrounding ecosystems. The findings also point to the limited capacity of conventional wastewater treatment to adequately control these contaminants and support the development of more effective treatment and management strategies to reduce their environmental dissemination. In line with approaches already reported internationally, this study provides new data from a still under documented local context and suggests, without establishing transfer pathways, environmental dissemination of anticancer residues as well as incomplete removal by conventional treatment processes.

Presence of anticancer drugs in groundwater

The results for the groundwater sample S4 reveal the presence of CP, IF, and 5-FU at notable MECmax concentrations (95.72 µg·L^{-1}; 72.267 µg·L^{-1}; 26.436 µg·L^{-1} respectively). DOX was detected at a low concentration of 5.989 µg·L^{-1}, whereas ETO was not detected or below the limit of detection (<LOD). A review of the literature reveals that this may be a few studies detect the presence of anticancer drugs in groundwater (López-Serna et al., 2013; Negreira et al., 2013; Nassour et al., 2020) (see Figure 4). CP,IF, 5-FU have been recognized for their persistence to biodegradation and high mobility, which may help explain their detection at elevated concentrations (Fonseca et al., 2017; Ghafuria et al., 2018). Conversely, doxorubicin and ETO appears to be predominantly accumulated in sediments (Évaluation Préalable Finale Pour Le Défi Concernant Le Numéro de Registre Du Chemical Abstracts Service Environnement Canada, 2008; Azuma, 2018). The obtained findings reveal that the chemical composition of groundwater closely mirrors that of targeted hospital effluents, highlighting the presence of a contamination plume originating from the healthcare facility. This scenario suggests the existence of previously underestimated preferential pathways, which may include leaking pipelines, drainage systems, or direct infiltration. The lithostratigraphic and dynamic characteristics of the Naima-Beni Oukil plain and the Bouhouria-Naima aquifer. This area is composed of a sequence of marls and calcareous conglomerates. In the upper layers, there is an alternation of stratocroissant and granocroissant structures, suggesting synsedimentary tectonic activity. The semi-permeable nature of the marls, combined with the topography and geological dynamics, has led to the formation of fissures within the strata (Jadid, 1999). The fissures permit the flow of effluent from the oncology center, which contains anticancer drugs, from septic tanks (S1)

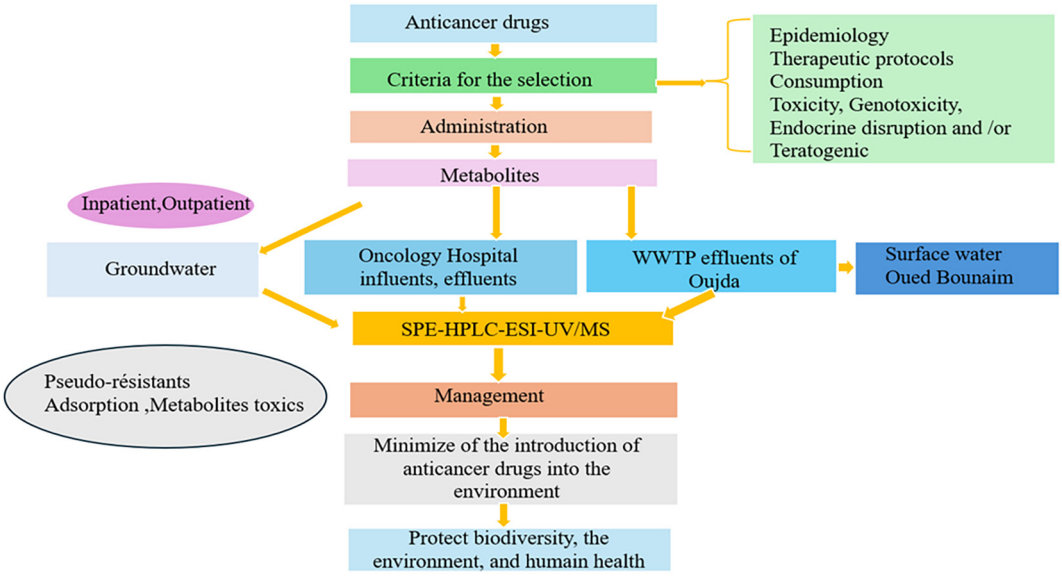

Figure 6. Pathways of anticancer drugs in the aquatic compartments

and (S2) into the groundwater (S4). Although based on a single well, the detection of anticancer drugs in groundwater indicates the need for further investigation of potential contamination of subsurface water resources, especially in the areas where groundwater contributes to drinking water supply.

CONCLUSIONS

This study provided new insight into the occurrence of anticancer drugs in aquatic matrices from the Oujda region and reveals a contamination pattern involving hospital effluents, groundwater near the hospital, and treated wastewater from the WWTP reused for irrigation. The detection of five anticancer drugs across these compartments suggests environmental dissemination of these residues, with concentration differences likely related to the physicochemical and pharmacokinetic properties of individual compounds. Their occurrence in groundwater near the hospital is compatible with a possible influence of hospital effluents, whereas their presence in treated wastewater used for irrigation points to a potential transfer to agricultural environments. These findings further suggest that conventional treatment processes may not achieve complete removal of these contaminants, and that adsorption may play a role in their environmental retention. Overall, the results highlight the need for improved hospital effluent management, reinforced groundwater monitoring, and broader investigations of anticancer drug residues in aquatic environments. While the study provided a preliminary contribution to understanding the environmental behavior of these compounds and supported their greater consideration in environmental assessment and management, the findings should be interpreted in light of several limitations. The work was restricted to a limited number of parent compounds, without accounting for metabolites or transformation products. Groundwater was represented by a single grab sample because of access constraints, limiting conclusions on spatial and temporal variability. Future studies should therefore expand the spatial and seasonal sampling design, incorporate environmental risk characterization, and assess the potential transfer of residues to crops irrigated with contaminated treated wastewater.

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