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Abstract

The photochemical behaviour of etodolac **was** investigated under various irradiation conditions. Kinetic data **were** obtained **after irradiation of** 10^{-4} M aqueous solutions by **UVB, UVA and direct exposure to sunlight. The Xenon lamp irradiation was used in order to determine the photodegradation quantum yield under sun-simulated condition (ϕ_{sun}). The value was determined to be = 0.10 ± 0.01.** In order to **obtain** photoproducts and for mechanistic purposes, experiments **were** carried out on more concentrated solutions by exposure to sunlight and to UVA and UVB lamps. **The drug underwent photooxidative processes following an initial oxygen addition to the double bond of the five membered ring and was mainly converted into a spiro compound and a macrolactam.** Ecotoxicity tests **were performed** on etodolac, its photostable **spiro derivative** and its sunlight irradiation mixture on two different aquatic trophic levels, plants (algae) and invertebrates (rotifers and crustaceans). Mutagenesis and genotoxicity **were** detected on bacterial strains. The results **showed** that only etodolac had long term effects **on** rotifers although at concentrations far from environmental detection values. A mutagenic and genotoxic potential **was** found for its derivative.

Keywords: etodolac, photooxidation, indoles, acute and chronic toxicity, mutagenesis, genotoxicity.

1. Introduction

Light and oxygen are two of the most important abiotic factors involved in the environmental fate of xenobiotics, in aquatic systems and/or in the surface of soils. **They received particular attention over the last decade (Brigante et al., 2005;** Neilson and Allard, 2008; Lambropoulo**u** and Nollet, 2014). **Direct or photosensitized transformation in natural aquatic media** can promote the **complete degradation** of the molecule, but in some cases leads to transformation products that can be more toxic and/or noxious than the parent compounds (Lambropoulo**u** and Nollet, 2014; Sinclair and Boxall, 2003).

Among the xenobiotics found in the environment, in the last years pharmaceuticals and personal care products (PPCPs) have been frequently detected worldwide (Fick et al., 2010; Negreira et al., 2014). After their consumption, drugs are eliminated as parent compounds and/or metabolites through human excreta reaching sewage treatment plants (STPs). These plants are often not able to completely remove such xenobiotics which may escape removal processes and end in the aquatic compartment in the range of sub-ng/L – µg/L. **Hence, phototransformation may be considered a more significant process compared to biodegradation in sunlit waters.** Unpredictable effects can be also associated to the derivatives obtained by **abiotic** transformations and, on the basis of **this evidence**, the evaluation of the potential adverse effects of drugs **and derivatives** is of increasing scientific concern (Jjemba, 2006; Isidori et al., 2009).

Among the most studied pharmaceuticals, the therapeutic group of nonsteroidal anti-inflammatory drugs (NSAIDs) is receiving great attention due to the worldwide use and frequent environmental occurrence. **Many of these compounds have shown a significant photoreactivity, often associated to phototoxic side effects (Bosca et al. 2001). NSAIDs have not been detected in aquatic systems at concentrations able to cause acute toxic effects in non-target organisms; however, studies on chronic and genotoxic effects, as well as the fate of parent compounds and their derivatives, are still lacking.**

We therefore focused our study on the drug etodolac (ETD), a member of the pyranocarboxylic acid group of NSAIDs. Approved by the U.S. Food and Drug Administration in 1991, it is used to treat mild to moderate pain, and to reduce inflammation associated to osteoarthritis and rheumatoid arthritis (Jones, 1999). **In 2011 it was included in the list of high production volume pharmaceuticals that were not detected in the environment but were estimated to be persistent and/or bioaccumulated (Howard and Muir, 2011). However, in the same year the drug was detected in river waters at concentration of 0.3 ng/L (Hoshina et al., 2011).** The drug is metabolized in the liver, and several metabolites, **such** as hydroxylated and glucuronide **derivatives**, have been identified in human plasma and urine (Strickmann and Blaschke, 2001). **Products deriving from decarboxylation and ring opening have been found in aqueous solutions at high temperature (75-95 °C) at different pH values (Lee et al., 1988).** To our knowledge, **data on** the photoreactivity of etodolac **have not been** reported in literature. The drug **ETD (Figure 1) contains, in its structure, the indole system that is a chromophore present in many molecules of biological interest, in such drugs as indomethacin (Wu et al.1997; Temussi et al, 2011), fluvastatin (Cermola et al. 2007), and vinblastine sulfate (Black et al. 1988) which show photoreactivity. This study aimed to evaluate the photochemical behaviour of etodolac under photochemical conditions with particular attention to identify the main photoproducts and elucidate the photolysis reaction pathway.** Parent compound and derivatives were tested for their aquatic toxicity in algae, rotifers and crustaceans. The unicellular green alga *Pseudikirchneriella subcapitata*, the freshwater rotifer *Brachionus calyciflorus* and the microcrustacean *Ceriodaphnia dubia* (Cladocera, Crustacea) were selected as representative aquatic organisms since they have a widespread geographic distribution and a strong impact on several important ecological processes in waters. The mutagenesis and genotoxicity were evaluated with and without metabolic activation (S9) through the salmonella mutagenicity assay in TA98 and TA100 *Salmonella typhimurium* strains and the UMU test in the *Salmonella typhimurium*

TA1535/pSK1002 to detect point mutations and to induce the *umu*C-gene in response to genotoxic lesions in the DNA, respectively.

2. Materials and Methods

2.1 Chemicals

Etodolac (**ETD**, **1**), (±)-1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-β]indole-1-acetic acid) was supplied by Kemprotec. It was at least 98% pure. All chemicals were used without further purification unless otherwise indicated. Solvents were of HPLC grade and they were supplied by Sigma- Aldrich. Water was of Milli-Q quality and was obtained from a Milli-Q gradient system (Millipore).

For toxicity and genotoxicity tests the **photoproducts (see section 2.5)** were dissolved in dimethylsulfoxide (Sigma-Aldrich Chemicals) because of their low solubility in water, **sonicated at 40 kHz for 20 min at room temperature by using an ultrasonic bath (Sonica®, SOLTEC, Milan, Italy)**, and further diluted in double-deionized water to obtain stock solutions. The test solutions were prepared by mixing the appropriate volumes of the stock solutions and the test media. **In the test solutions, DMSO percentage was lower than 0.02% v/v. For each test a solvent control was performed.**

2.2 Analytical instruments

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova-500 instrument operating at 499.6 and 125.6 MHz for ^{1}H and ^{13}C, respectively, and referenced with deuterated solvents ($CDCl_3$ or CD_3OD). The carbon multiplicity was evidenced by distortion-less enhancement by polarisation transfer (DEPT) experiments. The proton couplings were evidenced by ^{1}H-^{1}H COSY experiments. The heteronuclear chemical shift correlations were determined by HMQC and HMBC pulse sequences.

IR spectra were recorded on a Jasco FT/IR-430 instrument equipped with single reflection ATR using $CHCl_3$ as solvent.

UV–Vis spectra were recorded with a Varian Cary 300 UV–Vis spectrophotometer or on a PerkinElmer Lambda 7 spectrophotometer.

pH was measured with a Metrohm combined glass electrode, connected to a Metrohm 713 pH-meter.

HPLC-UV was an Agilent 1100 Series binary pump HPLC equipped with a UV detector set at 254 nm. The column used was a Gemini C18 (5µm, 250 mm x 4.6 mm**, Phenomenex) with a f**low rate **of** 0.4 mL min^{-1}**. The m**obile phase **was:** water with 0.1% formic acid (A) and acetonitrile (B). The gradient elution was: at initial time B 30 % and A 70%, followed by a linear gradient to **B** 75 % within 55 min, the ratio was maintained constant for 5 min, then the ratio returned to B 30% in 5 min.

LC-HR-MS analysis was carried out on an LC-MS system (Agilent 1100 Series, binary pump) equipped with an ion trap mass selective detector (MSD VL) and a UV diode array detector. The adopted column was a Sphere Clone C18 (4.6 µm, 250 mm x 5 mm, Phenomenex) and the gradient elution was 5% acetonitrile and 95% water followed by a linear gradient to 95% acetonitrile within 30 min, keeping constant the latter conditions for a further 15 min. The flow rate was 0.4 mL min^{-1}.

Analytical and preparative TLCs were made on Kieselgel 60 F_{254} plates with 0.2 mm, 0.5 or 1 mm layer thickness, respectively (Merck).

2.3 Irradiation set-up

All irradiation experiments were performed **in H_2O-CH_3CN (9:1 v/v) mixture (to have clear solution), except when specified, and were performed** in triplicate. **Solutions at 1 mM concentration were used to isolate photoproducts, whereas concentrations between 0.1-0.5 mM were choose for kinetic analyses.**

Three different kinds of lamps (UVA, UVB and Xenon lamp) and two irradiation set-up **(system I and system II)** were used **in order to investigate the degradation of etodolac (100-500 µM) and separate the photoproducts for the spectroscopic characterization and ecotoxicological tests (1 mM)**.

System I: a thermostated cylindrical reactor of 40 **mL**, cooled by water circulation at a temperature of 15 ± 2°C in order to limit thermal reactions, was located at one focal point of the a Xenon lamp (solar simulator) in order to maintain a constant irradiation of the whole sample and was equipped on the top with a pyrex filter removing the wavelengths lower than 285 nm. Samples were continuously stirred with a magnetic stirrer to ensure homogeneity. **Emission spectrum of lamp was measured with an Ocean Optics SD 2000 CCD spectrophotometer (calibrated using a DH-2000-CAL Deuterium Tungsten Halogen reference lamp).** The incident photon flux **in the UV region (285-400 nm) was quantified to be 34.4 W m^{-2}. The Xenon lamp irradiation experiment was performed in order to estimate the degradation rate of ETD and polychromatic degradation quantum yield under sun-simulated conditions.**

For the UVB and UVA irradiation (system II) a photoreactor (Multirays, Helios Italquartz) equipped with six 15W lamps with a maximum at 313 nm (UVB) or with four 15W lamps with a maximum at 366 nm (UVA) **was used**.

Finally, a solution of ETD was exposed to sunlight in H_2O-CH_3CN (9:1 v/v) (0.5 mM) in a closed quartz tube and samples were withdrawn in order to follow the ETD disappearance and the formation of main photoproducts under naturally sun irradiation conditions.

2.4 ***Kinetics constant*** *and quantum yield* ***determination***

The time evolution of etodolac **was** fitted with a pseudo-first order equation $C_0 = C_t\, e^{-kt}$ where C_0 was the initial drug concentration, C_t the concentration at time t and k the pseudo-first order degradation rate constant. Assuming the drug as the only absorbing species present in water, the

polychromatic quantum yield degradation (Φ) was calculated in the overlap range of etodolac absorption spectrum with lamp emission spectrum, as follows:

$$\Phi = \frac{R_x^d}{I_a} \qquad \textbf{(eq 1)}$$

where R_x^d is the drug degradation rate (M s^{-1}) and I_a is the absorbed photon flux *per* unit of surface and unit of time. The latter was calculated from:

$$I_a = \int_{\lambda_1}^{\lambda_2} I_0(\lambda)(1-10^{-\varepsilon(\lambda)d[X]d\lambda}) \qquad \textbf{(eq 2)}$$

where I_0 is the incident photon flux, ε is the molar absorption coefficient of xenobiotic, *d* the optical path length inside the cells and [X] the initial etodolac concentration. **λ_1 and λ_2 represent the interval of integration, that is the overlap range between etodolac absorption and lamp emission spectrum (λ_1 = 285 nm and λ_2 = 310 nm).**

Photoproducts were isolated and purified and the calibration curve was performed in HPLC-UV in order to follow their formation during ETD disappearance.

2.5 Photoproducts isolation

In order to isolate and characterize the main photoproducts, two solutions of etodolac at 1 mM were irradiated. The first one (50 mg of ETD) was exposed to sunlight (October 2013, Naples) for 3 days. After evaporation of the solvents, the mixture was separated by preparative TLC. Elution with Et_2O gave compound 2 (7 mg), etodolac (1 mg), compound 3 (11 mg) and an intractable material (12 mg).

The second solution (50 mg) was irradiated by UVB lamps for 1 h, then irradiation mixture was handled as reported before. The chromatographic separation gave compound 2 (30 mg), etodolac (8 mg) and an intractable material (8 mg). ETD and compounds 2 and 3 were fully characterized and all data are reported in Table 1.

***2.6** Ecotoxicity tests*

Aquatic toxicity **was performed on pure etodolac** (**1**), its isolated photostable **spiroderivative 2** and its **crude** sunlight irradiation mixture **(obtained after three days exposure)**. **Each dried test sample was firstly dissolved in DMSO and further diluted in double-deionized water. The toxicity** was determined on *Pseudokirchneriella subcapitata*, *Brachionus calyciflorus* and *Ceriodaphnia dubia*.

***2.6.1** Acute toxicity*

The *B. calyciflorus* (ASTM E1440-9, 2004) test was performed on organisms less than 2 h old that hatched from cysts supplied by MicroBioTest Inc. (Nazareth, Belgium). The organisms were hatched 16–18 h before the beginning of the test, in synthetic freshwater (moderately hard medium), at 25 ± 1 °C and under continuous illumination (3000–4000 lux). Tests were performed in multiwell plates, 0.3 mL of test solution (five concentrations following a geometric progression of 2) was tested in six replicates with five organisms per well.

The *C. dubia* test was performed over 24 h of exposure using young organisms less than 24 h old and following EPA-600-4-90 (applied to reference toxicant) procedures (US EPA, 1993). Organisms were hatched from ephippia (MicroBioTest) after 3–4 d of incubation under a light source of 6000 lux at 25 ± 1 °C in synthetic ISO medium. Tests were performed in 24-well plates with 10 crustaceans per well (1.0 mL of test solution), five concentrations and three replicates per concentration.

For each test, plates were incubated in darkness at 25 ± 1 °C for 24 h. Mortality was the test parameter and the concentration that induced the 50% effect compared to negative control was indicated as median lethal concentrations, LC50.

2.6.2 *Chronic toxicity*

The test on *P. subcapitata* was performed in 96-well microplates according to Paixao et al. (2008). The algal cells were collected from an exponentially growing pre-culture according to ISO 8692 (2004). The compounds (five concentrations, geometric progression 2) and the negative control were incubated with 10^4 cells/mL of algal suspension in six replicates; the final test volume was of 0.3 mL per well. The plates were incubated under continuous illumination (light source 6000 lux) at 25 ± 1 °C on a microplate shaker (450 rpm). The plates were read at 450 nm (Spectra fluor, Tecan, Switzerland) immediately before the test and every 24 h for 72 h.

The chronic toxicity test on *B. calyciflorus* was performed according to ISO 20666 procedure (ISO, 2008). Organisms, less than 2 h old, were hatched in the same conditions of acute assay. The tests were performed in multiwell plates, 0.9 mL of test solutions (five concentrations, geometric progression 3) were tested in six replicates with one rotifer per well. Organisms were fed with 0.1 mL of *P. subcapitata* fresh suspension (10^7 cells/mL). Plates were incubated in the dark at 25 ± 1 °C for 48 h.

The population growth inhibition was the test parameter considered for both chronic assays and the concentrations that gave the 50% effect compared to negative control were indicated as median effective concentrations, EC50.

2.6.3 *Mutagenesis/genotoxicity tests*

The salmonella mutagenicity assay was performed without metabolic activation (S9) on TA98 and TA100 *Salmonella typhimurium* strains. The TA98 strain was utilized to evaluate frame-shift mutations, while **the** TA100 **was used to assess** base-pair substitutions. Five compound concentrations (0.1 mL) and 0.1 mL of medium containing 10^8 cells were incorporated into agar

plate with 0.5 mL phosphate buffer to evaluate the direct mutagenicity. Three plates for each concentration were incubated at 37 °C for 72 h in the dark and then the number of His^+ revertants was counted. Sodium azide **(5, 10 and 20 µg/mL)** for TA100 and 2-nitrofluoren **(2.5, 5 and 10 µg/mL)** for TA98 were used as positive controls. The test parameter considered was Mutagenic Ratio (MR) calculated as the ratio between the mean number of revertants/plate of compound and the spontaneous revertants of the negative control. A compound was considered mutagenic when the MR was >2 with a clear dose–response relationship (EPA-600/4-82-068, 1983).

Genotoxicity was assessed by the *umu*-test according to the ISO 13829 procedure (ISO, 2000). The *umu*-test is a colorimetric assay based on the ability of genotoxic agents to induce the *umu*C-gene expression in the *Salmonella typhimurium* TA1535/pSK1002 strain in response to genotoxic lesions in the DNA. The *umu*C-gene expression can be easily monitored by β-galactosidase activity since the test strain contains the *umu*C-gene fused with the *lac*Z-gene. The assay was performed in 96-well microplates with and without S9 mix (0.45 mL of S9 fraction was added to 15 mL of bacterial suspension) to detect indirect and direct genotoxic effects. Five concentrations were tested in six replicates; 4-nitroquinoline N-oxide **(0.05 µg/mL)** and 2-aminoanthracene (**2 µg/mL)** were used as positive controls for direct and indirect genotoxicity, respectively. The production of β-galactosidase was assessed measuring the absorbance at 420 nm (Spectrafluor; Tecan, Mannedorf, Switzerland). Bacterial growth was measured at 600 nm and the Growth factor (G) was determined as $(A_{600,T} - A_{600,B})/(A_{600,N} - A_{600,B})$ where $A_{600,T}$ = absorbance of the sample well at 600 nm; $A_{600,N}$ = absorbance of the negative control at 600 nm and $A_{600,B}$ = absorbance of the blank at 600 nm. The test parameter considered was Induction Ratio $(IR)=1/G\cdot[(A_{420,T} - A_{420,B})/(A_{420,N} - A_{420,B})]$ where $A_{420,T}$ = absorbance of the sample well at 420 nm; $A_{420,B}$ = absorbance of the blank at 420 nm; $A_{420,N}$ = absorbance of the negative control at 420 nm. A compound was considered genotoxic when IR value was > 1.5 with an evident dose–response effect.

3. Results and Discussion

UV-**v**is spectrum of etodolac **(ETD) at pH 2.0, 7.0 and 10.0 is reported in Figure 1 showing that any significant variation could be appreciated over a large range of pH values. Etodolac spectra show two absorption bands centred at λ 225 nm and 272 nm with molar absorption coefficients of 42600 and 8290 M^{-1} cm^{-1}, respectively.**

Preliminary experiments to test the stability of the drug in the dark were performed at room temperature **at various environmentally relevant pH (from 4.0 up to 9.0) (Valenti et al., 2009) showing that any significant transformation can be appreciated after 24 hours.**

From preparative irradiations of ETD under UVB and sunlight two main photoproducts were isolated, characterized by NMR, mass and structures are reported in Table 1. For the photoproduct 2 LC-HR-MS analysis presents a molecular peak at 304.1471 *m/z* $[M+H]^+$ (with M corresponding to M_{drug} + O), the peaks at 286.1611 *m/z* $[M-OH]^+$ and *m/z* 244.1440 $[M-CH_2COOH]^+$. ^{13}C-NMR analysis shows the presence of a signal at δ 181.2 due to the ketone function (C-3) and a signal at δ 60.8 assigned to the spiro carbon (C-2). HMBC spectrum evidences correlations of H-4' methylene protons with C-1', C-3' and carbonyl carbon (C-3). The methylene protons of the acetic acid side chain (C-5'), in the HMBC experiment, give heterocorrelations with the C-1' quaternary carbon, the carboxylic carbon (C-6'), and C-2 spiro carbon.

For the photoproduct 3, the LC-HR-MS analysis shows a molecular peak at 320.2069 *m/z* $[M+H]^+$ (with M corresponding to M_{drug} + 2 O) and fragmentation peaks at 302.2107 *m/z* $[M-OH]^+$ and 260.2204 *m/z* $[M-CH_2COOH]^+$. ^{13}C-NMR analysis shows the presence at downfield of a carbonyl carbon at δ 204.6 (C-3) and an amidic carbon at δ 172.0 (C-2). HMBC spectrum evidences correlations of the carbonyl carbon (C-3) with protons at δ 4.45 and 4.16 assigned to the oxymethylene group (C-3'). The methylene protons of the

acetic acid function (C-5'), in the HMBC experiment, give heterocorrelation with the quaternary carbon at δ 80.7 (C-1') and the carboxylic carbon at δ 177.5 (C-6').

As reported in Figure 2, the effect of UV radiation was investigated on the ETD degradation and generation of photoproducts 2 and 3. Under UVB the main photoproduct is compound 2. After 30 min of irradiation about 70 % of ETD (corresponding to 300 µM) is degraded into spiro compound 2 (200 µM), while lactam 3 formation occurs only at smaller amount (~30 µM).

Under UVA irradiation 65% degradation of ETD occurs after 360 min (about 370 µM) and concentrations of spiro 2 and lactam 3 are about 150 and 180 µM, respectively.

Finally, a drug solution was exposed to sunlight and it was observed a half-life time of about one day (~11 hours of light). The degradation kinetics for sun radiation were calculated considering that the daylight in October (Naples, Italy) is in average of 11 hours for day. As shown in Figure 2, during the first hours of irradiation the photoproduct 3 is rapidly formed, after one day its concentration slowly decreases until complete degradation. A different behaviour is observed for photoproduct 2: its initial formation rate is slower than that of product 3, but it is photostable and after the complete etodolac degradation its concentration (~250 µM) remains almost unchanged for two weeks. Initial degradation rate of ETD (R_{ETD}^{d}, M s^{-1}) has been quantified to be 2.74 ± 0.12 × 10^{-7} M s^{-1} and 3.40 ± 0.06 × 10^{-8} M s^{-1} under UVB and UVA, respectively. Using Xenon lamp irradiation the R_{ETD}^{d}, M s^{-1} was 1.06 ± 0.03 × 10^{-8} M s^{-1} giving a polychromatic quantum yield ϕ in the UV-region of 0.10 ± 0.01. Interestingly, the degradation rate was close to those estimated using natural sun radiation (6.18 ± 0.19 × 10^{-8} M s^{-1}).

As reported in Figure 3, after ~90 min etodolac (25% of degradation) is converted to photoproduct 3 for 20% and compound 2 for 5% . These ratios are inverted when the drug is completely degraded, in this case a formation of 40% for 3 and 47% of 2 is observed.

All these results highlight the strong dependence of photoproduct distribution on irradiation wavelengths. For example, after 50% of degradation, etodolac is converted in compound 2 for 17% (UVA), 11% (sun), 42% (UVB), and in compound 3 for 33% (UVA), 39% (sun), 8% (UVB) (Figure 3). The most important differences are observed between UVA and UVB, the photoproduct distribution for sunlight exposure is more similar to UVA than UVB distribution due to the high contribute of UVA light to sun spectrum.

3.1 Mechanistic hypothesis

Photoproducts 2 and 3 are oxygenated compounds and this highlights the oxygen involvement in the etodolac transformation. These compounds are not produced under de-areated conditions. Indeed, experiments showed that when the drug (1 mM) was irradiated with UVA or UVB lamps under argon atmosphere, it was recovered almost unchanged even after one hour. Irradiation in aerated acetonitrile was equally slow but the formation of compounds 2 and 3 were observed showing that water is not determinant in their formation.

Rearrangement reactions to form spiro compounds from indoles have been sometime observed in photooxygenation reactions of indoles (Iesce et al., 2005) and in some cases the related intermediates have also been evidenced (Mateo et al., 1996). Moreover, this kind of products (spiro compounds) are found mainly in the photooxygenation of indoles where the pyrrolic ring is condensed with hexatomic hetero- (Mateo et al., 1996) or carbocycle (Cermola et al., 2007), like in etodolac. Analogously, formation of products with oxidative heterocyclic ring breakage, as photoproduct 3, has been reported (Cermola et al., 2007; Temussi et al., 2011). According to literature and to our data, a plausible phototransformation pathway is depicted in Scheme 1. The excited drug should

undergo oxygen attack to C2-C3 bond leading, via diradical 4, to hydroperoxide 5; the latter should give the corresponding alcohol 6 via homolysis followed by H abstraction from the solvent (Dussault, 1995) or, mainly, via water involvement (Mateo et al., 1996). As proved by Mateo et al. (1996), alcohol 6 in the presence of a weak acid (all species have a carboxylic acid group) should rearrange to photoproduct 2. Both diradical 4 and hydroperoxide 5 could lead to dioxetane 7, that through the well-known C-C and O-O bonds cleavage should give lactam 3 (Iesce et al., 2005).

The proposed reaction pathway is supported by literature data and also by LC-MS analysis of the irradiation mixture (after 10 min UV-B irradiation, 0.5 mM). The LC-HR-MS data evidenced the presence of 1, 2 and two intermediate compounds with $[M+H]^+$ peaks at 320.1463 and 304.1704 *m/z* that could be attributable to intermediates 5 and 6, respectively. They are unstable in our conditions and rapidly disappeared with increasing of the peak of spiro 2. Both compounds 5 and 6 were not recovered by chromatography due to instability and very low amounts.

Lactam 3 was found by sunlight exposure or UVA lamps. This should be due to these mild irradiation conditions that are favorable for UV-sensitive peroxidic and hydroperoxidic species (Iesce et al., 2005) and to the photosensitivity of lactam 3. In fact, irradiation experiments of photoproducts showed that lactam 3 (1 mM) was completely degraded within three hours by UVB light and in 15 days by sunlight exposure. Under the latter conditions spiro 2 remained unchanged over time up to 2 weeks.

3.2 *Ecotoxicological results*

Etodolac, its photostable derivative **2** and its sunlight irradiation mixture, obtained simulating the environmental photodegradation, were subjected to acute and chronic toxicity tests on organisms from two trophic aquatic levels. **Solvent controls showed no toxicity at the concentrations tested.** The results of acute toxicity tests, expressed as median lethal

concentrations (LC50) and chronic toxicity tests, expressed as median effective concentrations (EC50) are reported in Table 2. The range of concentrations tested for acute and chronic toxicity was in the order of mg/L, certainly higher than the etodolac environmental concentrations detected in the order of ng/L (Hoshina et al., 2011). As shown in Table 2, parent compound and its derivative were neither acutely toxic in rotifers and crustaceans up to 25 mg/L nor in the long-term exposure in algae up to 10 mg/L. Only etodolac induced a chronic effect expressed as 50% reproduction inhibition in *B. calyciflorus* at 15.44 mg/L. At the best of our knowledge, no ecotoxicity data exists for this drug **nor** for its derivative. Some studies, performed on the most detected NSAIDs in aquatic systems, demonstrated the low acute toxicity of such compounds in non-target organisms, with much higher short-term EC50 values than their environmental occurrence (Cleuvers, 2004; Parolini et al., 2012). Kawabata et al. (2013) studied sulindac and indomethacin, two NSAIDs of the indole acid class as etodolac, showing ecotoxicity values in bacteria in the same order of magnitude found for etodolac.

Although in the present study the phototrasformation process did not produce a toxic derivative, the sunlight-exposed samples show often differences in toxicity when compared to parent compound with formation of more or less hazardous derivatives for the environment and human health. Indeed, Schmitt-Jansen et al. (2007) evaluated the phytotoxicity of diclofenac, a NSAID belonging to the family of carboxylic acid derivatives, after exposure to natural sunlight. These authors demonstrated that the increasing concentration of transformation products significantly correlated with enhanced phytotoxicity. On the contrary Kawabata et al. (2013) reported that the photoproducts of sulindac and indomethacin were less toxic in bacteria than parent compounds. In the present research, the photo-exposed solution was also examined to detect possible additive, synergistic or antagonistic toxic

effects of the mixture photodegradation products and neither acute nor chronic toxicity was found.

In order to better determine the real environmental impact of etodolac, in our study we have also investigated the mutagenic and genotoxic properties of the parent compound, its photostable derivative **2** and the sunlight irradiation mixture. The mutagenesis results are shown in Table 3, where Mutagenic Ratios (MR) higher than 2 and a clear dose-related effect in TA98 strain was found for the photostable derivative and the mixture in the range of 6.25–25 and 12.5-25 μg/mL, respectively, suggesting that they are direct frame-shift mutagens. The photostable derivative was shown to be also a base-pair substitution mutagen in TA100, in the range of 6.25-25 μg/mL. Our results showed that the parent compound photodegradation leads to the formation of a derivative with mutagenic properties even if in the sunlight irradiation mixture the presence of other not identified photodegradation products reduced the activity of the photostable derivative (**2**) probably for antagonistic effects.

UMU-test results are shown in Table 4. Etodolac and sunlight irradiation mixture did not induce DNA damage with and without S9 mix, while the photostable derivative was found a direct genotoxic agent with an Induction Ratio (IR) higher than 1.5 starting from 3.125 mg/L. **In the UMU-test, the photoproduct showed higher activity than parent compound as it has been shown in the Salmonella mutagenicity assay. Furthermore, the mixture exhibited lower activity than photoproduct according to the Salmonella assay results probably due to an antagonistic effect exerted by other not identified photodegradation products. The** genotoxic properties of **the** derivative were totally suppressed by microsomal metabolic system.

Nowadays no data about mutagenicity and genotoxicity of etodolac as well as of photoproducts exists. However, the low activity of parent compound was confirmed by previous studies performed on different NSAIDs in which such compounds did not induce

gene mutations in *Salmonella typhimurium* strains and were not able to produce DNA damage at environmental concentrations (Philipose et al., 1997; Giri and Mukhopadhyay, 1998; Isidori et al., 2005; Parolini et al. 2011).

4. Conclusion

Photochemical fate of etodolac **was investigated under polychromatic wavelength (UVB, UVA and sun-simulated conditions). Moreover the** half-life **was estimated to be of about 1 day under** solar exposition. Two major photoproducts have been isolated and characterized **by NMR and HPLC-MS tools. Under UVA, both** spiro **compound (2)** and a macrocyclic lactam **(3) are generated while under UVB radiation mainly the compound spiro, found to be genotoxic, was observed. Moreover t**he spiro compound **results to be** photostable **under sunlight exposure** after **up to 2 weeks suggesting the possible accumulation in aquatic media.**

The work gives further information on the reactivity of indoles, a system widely present in natural bioactive molecules as well as in several synthetic drugs. In particular, it confirms the easy photoreactivity of heterocyclic pyrrole ring under oxidative conditions and the tendency of this heterocycle, especially when condensed to an aromatic ring, as in indoles, to generate radical oxygenated species. Photochemical studies of xenobiotics, in particular drugs, in addition to the environmental interest, aim at obtaining information on the photochemical reactivity of certain features present in these molecules (structure-photoreactivity relationship). Given the complexity and heterogeneity of drugs, it is difficult to predict or rationalize their behavior in the environment. On the other hand, the knowledge of factors (substituents, solvents) that affect the photochemical behavior of organic molecules are limited to relatively simple derivatives.

Our toxicological data evidenced that etodolac **and its photostable derivative** are not of particular environmental concern **as long as their** toxicity occurs at very high **concentrations, far above the detected environmental occurrence. Such results are consistent with the toxicity levels found**

for other NSAIDs. Nevertheless, the derivative exhibited mutagenic and genotoxic activity highlighting the need of investigating other relevant potential effects that to date are not clearly regulated by the environmental risk assessment guidelines.

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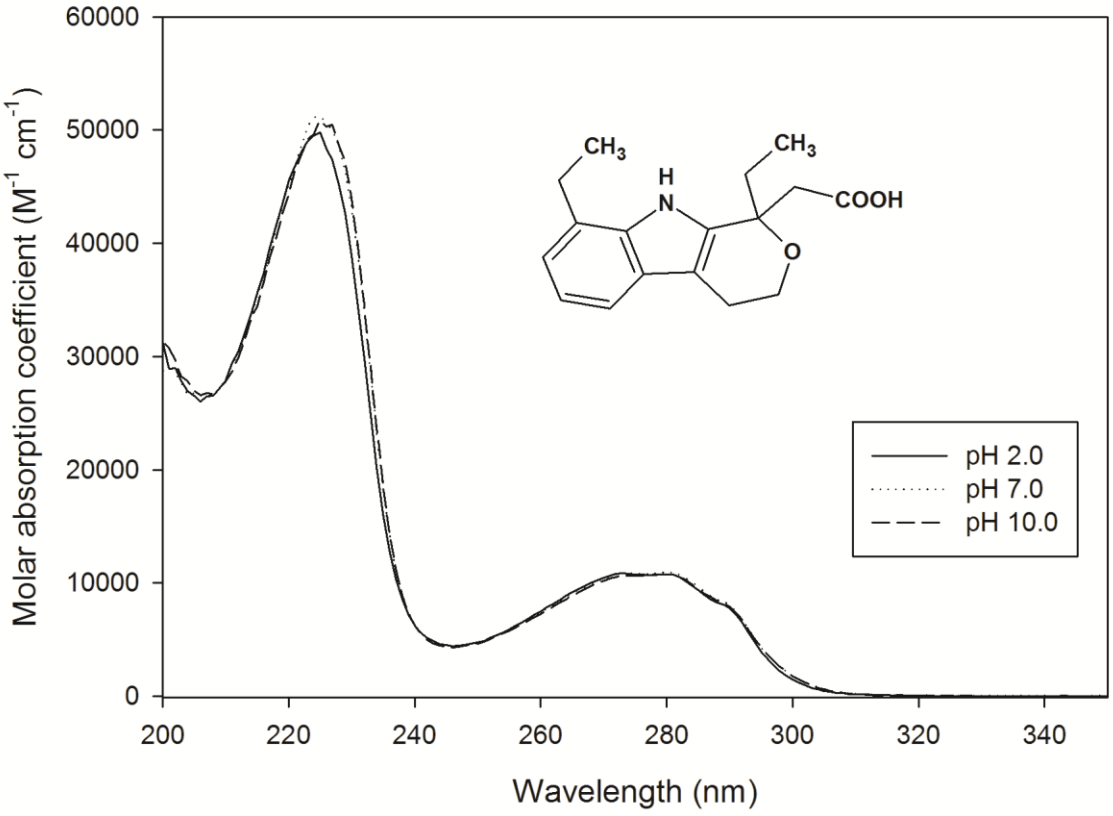

60000
50000
40000
30000
20000
10000
0
Molar absorption coefficient (M-1 cm-1)
200
220
240
260
280
300
320
340
Wavelength (nm)
CH3
CH3
H
N
COOH
O
pH 2.0
pH 7.0
pH 10.0

Fig. 1

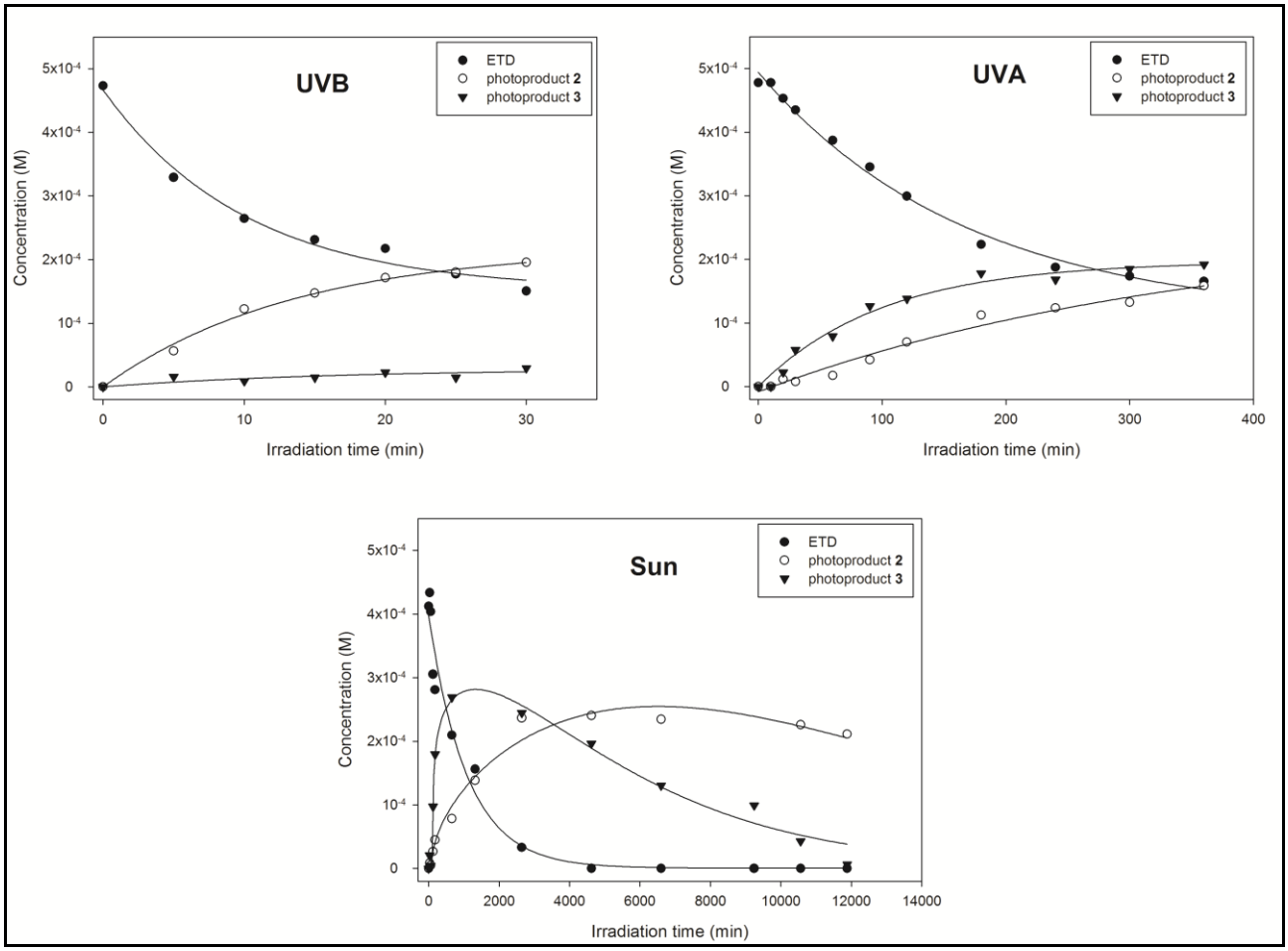

UVB
ETD
photoproduct 2
photoproduct 3
Concentration (M)
Irradiation time (min)
UVA
Sun

Fig. 2

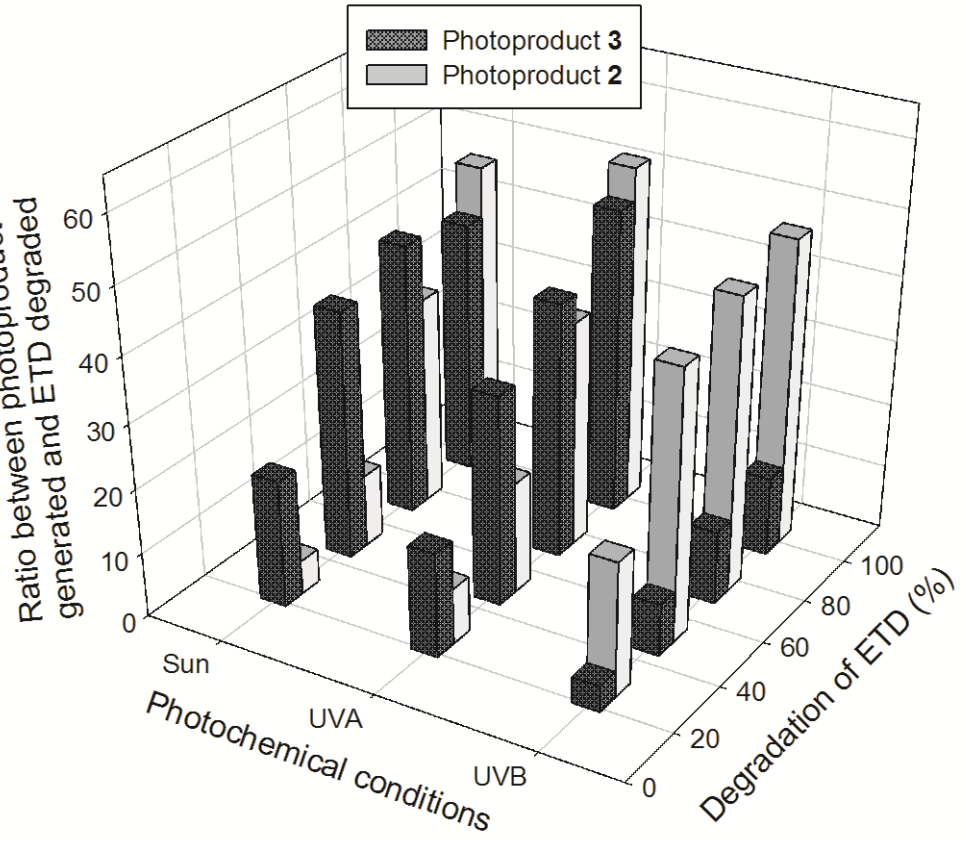

Photoproduct 3
Photoproduct 2
Ratio between photoproduct generated and ETD degraded
0
10
20
30
40
50
60
Sun
UVA
UVB
Photochemical conditions
0
20
40
60
80
100
Degradation of ETD (%)

Figure 3

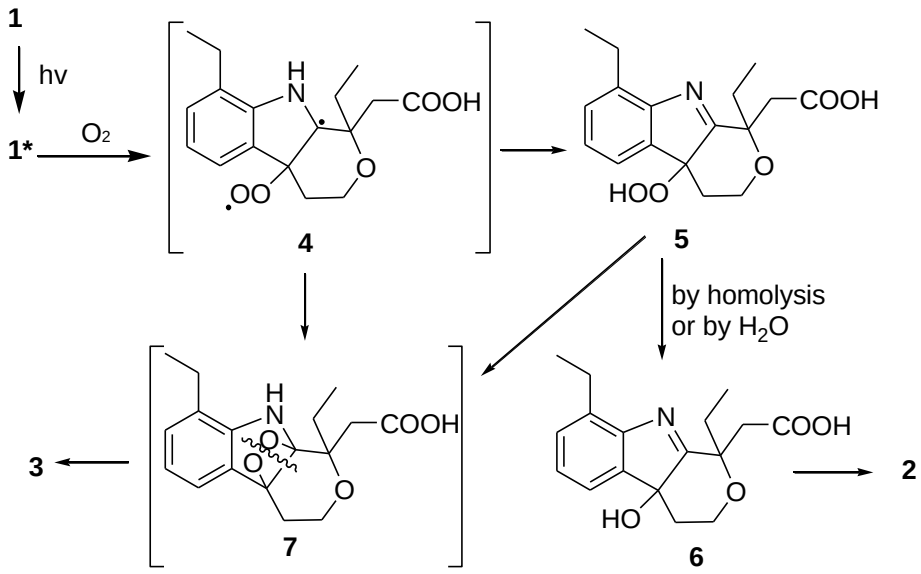
Figure Caption

Fig. 1. UV-vis spectra of etodolac (ETD) at different pH values in H_2O/CH_3CN mixture 9:1 (v/v). Insert chemical structure of etodolac.

Fig. 2. Degradation profiles of ETD and formation of photoproducts 2 and 3 as a function of different irradiation times under UVB, UVA and sun (autumn in Italy) irradiation.

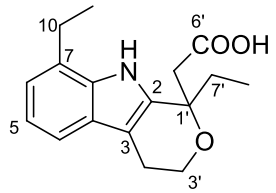
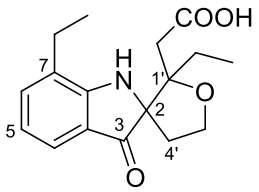
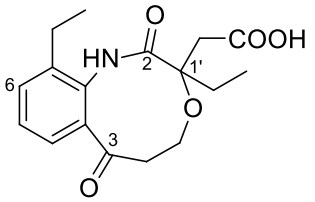
Fig. 3. Ratio of photoproducts 2 and 3 generation (z-label) as a function of ETD degradation (y-label) under UVB, UVA and sun irradiation (x-label).

Scheme 1. Suggested pathways for photoproducts 2 and 3.


1
hv
1*
O2
H
N
COOH
O
OO
4
N
COOH
O
HOO
5
by homolysis
or by H2O
H
N
O
O
COOH
O
7
3
N
COOH
O
HO
6
2

Scheme 1

Table 1. Spectral data of compounds **1-3**

	1 (ETD)		**2**		**3**	
						
Retention time (HPLC-UV)	41.3 min		24.5 min		20.2 min	
HR ESI-MS (*m/z*)	288.2082 $[M+H]^+$, 270.1903, 228.1623, 172.1372, 144.1033		304.1471 $[M+H]^+$, 286.1611, 244.1440, 188.1179		320.2069 $[M+H]^+$, 302.2107, 260.2204, 204.1767	
IR (CH_2Cl_2) ν (cm^{-1})	3430, 1721, 1077		3445, 3193, 2978, 1710, 1447		3380, 2952, 1720, 1698, 1645, 1452	
UV (H_2O:CH_3CN 9 :1 v/v) λ (nm)	225 (log ε 4.6), 272 (log ε 3.9)		255 (log ε 3.6), 225 (log ε 3.4)		286 (log ε 1.2), 340 (log ε 3.6)	
NMR[a]/ Position	δ_H[b]	δ_C	δ_H[b]	δ_C	δ_H[b]	δ_C
NH	4.62 (s)				7.98 (s)	
2		136.7 (q)[c]		60.8 (q)[c]		172.0 (q)[c]
3		116.5 (q)		181.2 (q)		204.6 (q)
4	6.90 (d, 6.5)	120.2(t)	7.08 (d, 7.6)	124.0 (t)	7.49 (d, 7.7)	124.7 (t)
5	6.93 (t, 7.0)	121.2 (t)	6.98 (t, 7.5)	124.1 (t)	7.39 (t, 7.6)	128.7 (t)
6	7.24 (d, 7.6)	121.2 (t)	7.12 (d, 7.4)	127.7 (t)	7.25 (d, 7.4)	132.1 (t)
7		127.8 (q)		133.7 (q)		142.8 (q)
8		136.2 (q)		140.3 (q)		138.9 (q)
9		127.9 (q)		129.4 (q)		133.5 (q)
10	2.86 (br q, 7.6)	25.1 (s)	2.62 (dq, 7.5, 1.0)	25.4 (s)	2.77 (dq, 7.5, 1.0)	30.5 (s)
11	1.30 (t, 7.6)	14.8 (p)	1.19 (t, 7.6)	15.4 (p)	1.26 (t, 7.5)	14.2 (p)
1′		77.0 (q)		89.9 (q)		80.7 (q)
3′	4.09 – 3.96 (m)	61.9 (s)	4.31 (td, 9.7, 4.1), 4.17 (dd, 16.4, 8.6)	65.9 (s)	4.45 (t, 12.0), 4.16 (ddd,13.5, 4.7, 2.1)	63.7 (s)
4′	2.83 – 2.63 (m)	32.2 (s)	2.78 – 2.69 (m), 2.14 (m)	39.7 (s)	3.17 (m), 2.47 (dd, 14.7, 3.4)	42.9 (s)
5′	3.00 (d, 14.3), 2.87 (d, 14.3)	44.0 (s)	3.01 (d, 14.3), 2.80 (d, 14.3)	38.0 (s)	3.18 (d, 14.6), 2.86 (d, 14.6)	35.9 (s)
6′		174.6 (q)		175.0 (q)		177.5 (q)
7′	2.11 (q, 7.3)	23.2 (s)	1.93 (m), 1.50 (m)	28.7 (s)	2.11 (dq, 14.4, 7.3) 1.96 (dq,	23.6 (s)

					12.7, 6.4)	
8′	0.74 (t, 7.3)	8.4 (p)	0.57 (t, 7.5)	9.0 (p)	1.13 (t, 7.5)	7.9 (p)

[a] Compounds **1** and **2** in CD_3OD, compound **3** in $CDCl_3$.

[b] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J* in Hz).

[c] Letters, p, s, t and q, in parentheses indicate, respectively, the primary, secondary, tertiary and quaternary carbons, assigned by HSQC and HMBC experiments.

Table 2. Ecotoxicity results in mg/L, expressed as L(E)C50 values with confidence limits (95%), of etodolac (**1**), its photostable derivative **2** and its irradiation mixture. Acute toxicity was assessed after 24h of exposure while chronic toxicity after 48h in *B. calyciflorus* and 72h in *P. subcapitata*.

	C. dubia LC50 (24h)	*B. calyciflorus* LC50 (24h)	*B. calyciflorus* EC50 (48 h)	*P. subcapitata* EC50 (72 h)
1	N.E. up to 25	N.E. up to 25	15.44 (5.27- 45.71)	N.E. up to 10
2	N.E. up to 25	N.E. up to 25	33% effect at 25	N.E. up to 10
Irradiation mixture	N.E. up to 25	N.E. up to 25	N.E. up to 25	N.E. up to 10

N.E.: No Effect

Table 3. Mutagenicity results expressed as Mutagenic Ratio (MR) of etodolac (**1**), its photostable derivative **2** and its irradiation mixture. The salmonella mutagenicity assay was performed on *S. typhimurium* TA98 and TA100 strains. In bold the MRs higher than 2.

	AMES TEST					
	TA98			TA100		
Compounds	Concentration (μg/mL)	Mean revertants/plate (± SD)	[1]MR	Concentration (μg/mL)	Mean revertants/plate (± SD)	[1]MR
Negative Control	-	50.5 ± 7.2	-	-	189.7 ± 52.6	-
2- Nitrofluoren	2.5	133.6 ± 13.6	**2.6**	-	-	-
	5.0	170.0 ± 25.7	**3.4**			
	10	351.3 ± 131.4	**6.9**			
Sodium Azide	-	-	-	5	361.0 ± 127.0	1.9
				10	618.0 ± 176.7	**3.2**
				20	862.3 ± 291.0	**4.5**
1	1.56	50.3 ± 10.0	1.0	1.56	194.0 ± 51.2	1.02
	3.125	52.0 ± 8.0	1.03	3.125	232.0 ± 50.0	1.2
	6.25	64.7 ± 1.5	1.3	6.25	198.0 ± 30.2	1.04
	12.5	59.0 ± 9.5	1.2	12.5	194.6 ± 69.2	1.02
	25	47.7 ± 15.3	0.9	25	272.0 ± 39.4	1.4
2	1.56	98.0 ± 25.9	1.9	1.56	189.0 ± 70.5	1.0
	3.125	143.2 ± 51.3	1.8	3.125	297.3 ± 96.5	1.6
	6.25	228.5 ± 120.0	**4.5**	6.25	349.7 ± 89.1	**2.1**
	12.5	552.3 ± 135.3	**10.9**	12.5	610.0 ± 181.5	**3.2**
	25	874.5 ± 203.3	**17.3**	25	1065.7 ± 214.7	**5.6**
Irradiation mixture	1.56	65.3 ± 8.9	1.3	1.56	218.3 ± 53.9	1.2
	3.125	66.7 ± 15.7	1.3	3.125	247.3 ± 35.8	1.3
	6.25	80.2 ± 19.7	1.6	6.25	229.3 ± 33.1	1.2
	12.5	117.1 ± 24.1	**2.3**	12.5	378.7 ± 58.3	1.9
	25	213.9 ± 60.9	**4.2**	25	287.0 ± 33.0	1.5

±SD = Standard Deviation obtained from three independent experiments.
[1] MR (Mutagenic Ratio): number of revertants/plate compared to the negative control

Table 4. Genotoxicity results expressed as Induction Ratio (IR) of etodolac (**1**), its photostable derivative **2** and its irradiation mixture. UMU-test was performed on the *S. typhimurium* TA1535/pSK1002 strain. In bold IRs higher **than 1.5**.

	UMU TEST			
	- S9		+S9	
Compounds	Concentration (μg/mL)	[1]IR	Concentration (μg/mL)	[1]IR
Negative Control	-	1.0		1.0
4-nitroquinoline N-oxide	0.05	3.71 ± 1.0		
2-aminoanthracene			2.0	4.71 ± 0.68
1	1.56	0.45 ± 0.21	1.56	0.51 ± 0.12
	3.125	0.72 ± 0.32	3.125	0.59 ± 0.11
	6.25	0.66 ± 0.21	6.25	0.50 ± 0.09
	12.5	0.66 ± 0.22	12.5	0.53 ± 0.11
	25	0.71 ± 0.32	25	0.56 ± 0.12
2	1.56	1.08 ± 0.41	1.56	0.44 ± 0.21
	3.125	**1.51 ± 0.32**	3.125	0.58 ± 0.19
	6.25	**1.92 ± 0.29**	6.25	0.68 ± 0.20
	12.5	**1.93 ± 0.39**	12.5	0.78 ± 0.21
	25	**1.96 ± 0.27**	25	0.62 ± 0.15
Irradiation mixture	1.56	0.96 ± 0.11	1.56	0.45 ± 0.18
	3.125	0.84 ± 0.31	3.125	0.79 ± 0.12
	6.25	0.88 ± 0.32	6.25	0.87 ± 0.19
	12.5	0.88 ± 0.33	12.5	0.98 ± 0.17
	25	1.38 ± 0.72	25	1.20 ± 0.32

±SD = Standard Deviation obtained from three independent experiments.
[1] IR (Induction Ratio):