Methodology
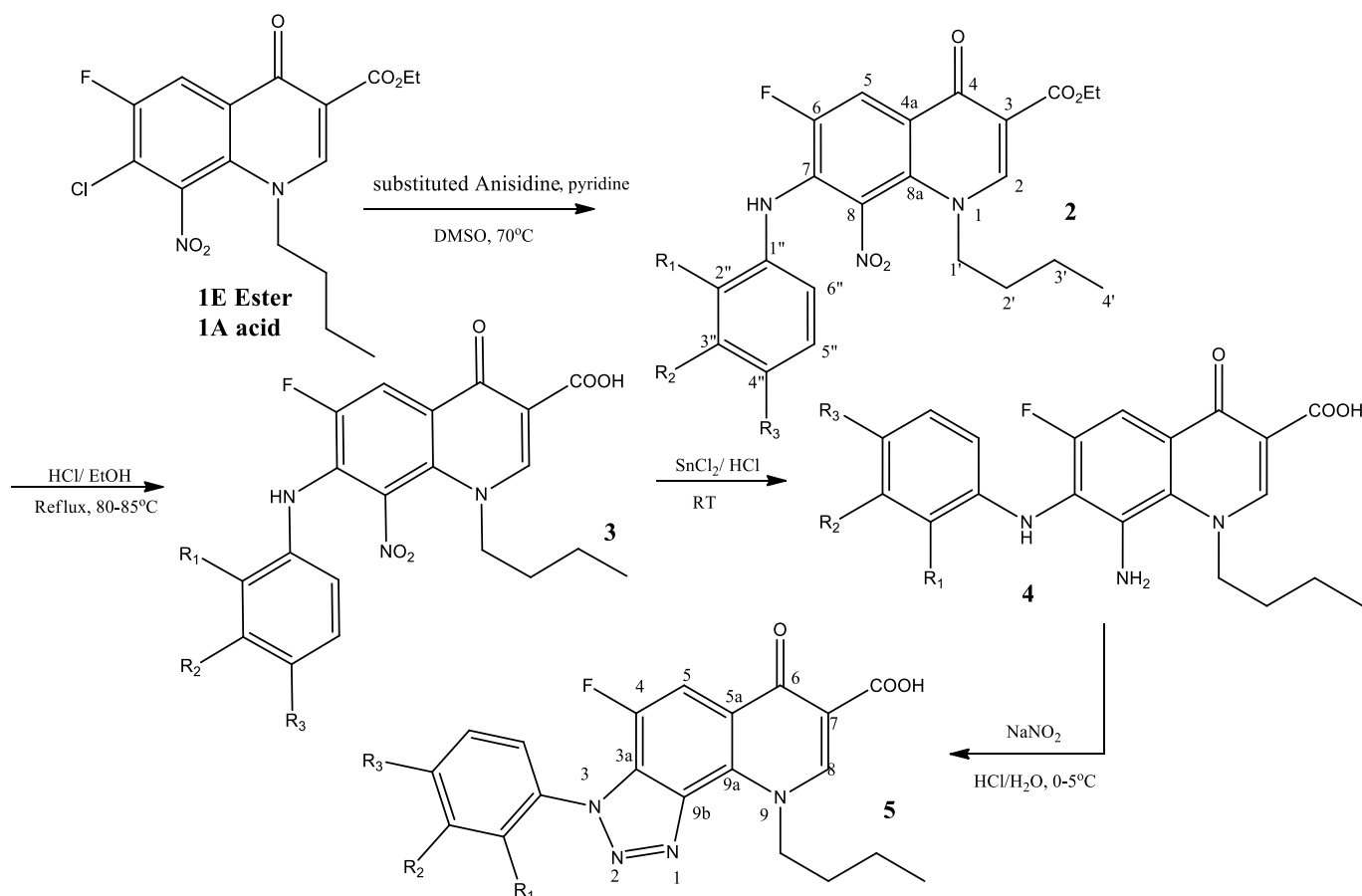## Chemistry

Fluoroquinolones tested in this work were prepared following Scheme 1, Table 1 (Alabsi et al, 2018; Elsheikhi, 2018; Al-Nuaimi, et al., 2021)

Scheme 1. Structure of Synthon 1-5 (a-e) and Table 1 for substitutions

Compound 2-5	Anisidine derivative	R_1	R_2	R_3	R_4
a	2-Methoxy aniline (o-anisidine)	OMe	H	H	H
b	3-Methoxy aniline (m-anisidine)	H	OMe	H	H
c	4-Methoxy aniline (p-anisidine)	H	H	OMe	H
d	2,4-Dimethoxy aniline	OMe	H	OMe	H
e	3,4,5-Trimethoxy aniline	H	OMe	OMe	OMe
f	4-Methyl aniline (p-toluidine)	H	H	Me	H

Tested FQs 2-5a were prepared according to Elsheikhi (2018) and Al-Nuaimi, et al., (2021) whereas compounds 2-5b-f were prepared according to Alabsi et al., (2018). All compounds were used as pure samples after verification over the TLC system using the following mobile phases mixtures were 94:5:1 chloroform-methanol-formic acid (CHCl3-MeOH-FA) (system 1) and 50:50 (n-hexane - Ethyl acetate) (system 2). Their structures were interpreted and checked. The structure of the active hit was fully characterized through this work to make sure that no oxidation or degradation occurred. The compounds were dissolved in DMSO to obtain a final concentration of 10 mg/mL (stock solution DMSO).

IUPAC Name of FQs prepared in scheme 1 (Table 1)
A-Starting material IUPAC names

- 1-Butyl-7-chloro-6-fluoro-8-nitro--4-oxo-1, 4-dihydroquinoline-3-carboxylic acid. (**1A**)
- Ethyl 1-butyl-7-chloro-1-cyclopropyl-6-fluoro-8-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylate. (**1E**).

B- Nitro acid derivatives 3a-f. Synthon 3

- 1-Butyl-6-fluoro-7-(2-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3a**)
- 1-Butyl-6-fluoro-7-(3-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3b**)
- 1-Butyl-6-fluoro-7-(4-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3c**)
- 1-Butyl-6-fluoro-7-(2,4-dimethoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3d**)
- 1-Butyl-6-fluoro-7-(3,4,5-trimethoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3e**)
- 1-Butyl-6-fluoro-7-(4-methyl-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3f**)

-C - Reduced acid derivatives 4a-f. Synthon 4

- 8-amino-1-butyl-6-fluoro-7-(2-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4a**)
- 8-amino-1-butyl-6-fluoro-7-(3-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4b**)
- 8-amino-1-butyl-6-fluoro-7-(4-methoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4c**)
- 8-amino-1-butyl-6-fluoro-7-(2,4-dimethoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4d**)
- 8-amino-1-butyl-6-fluoro-7-(3,4,5-trimethoxy-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4e**)
- 8-amino-1-butyl-6-fluoro-7-(4-methyl-phenylamino)-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**4f**)

D – Triazolo acid derivatives 5a-f. Synthon 5

- 9-Butyl-4-fluoro-3-(2-methoxy-phenyl)-6-oxo-6,9-dihydro-3H-[1, 2, 3] triazolo [4, 5-h] quinoline-7-carboxylic acid (**5a**)
- 9-Butyl-4-fluoro-3-(3-methoxy-phenyl)-6-oxo-6,9-dihydro-3H-[1, 2, 3] triazolo [4, 5-h] quinoline-7-carboxylic acid (**5b**)
- 9-Butyl-4-fluoro-3-(4-methoxy-phenyl)-6-oxo-6, 9-dihydro-3H-[1, 2, 3]triazolo [4, 5-h] quinoline-7-carboxylic acid (**5c**)
- 9-Butyl-4-fluoro-3-(4-methyl-phenyl)-6-oxo-6,9-dihydro-3H-[1,2,3]triazolo[4,5-h]quinoline-7-carboxylic acid (**5f**)

Anti-inflammatory (Nitrite) Determination in Vitro

RAW 264.7 mouse macrophage cell line (ATCC® TIB-71) were cultivated in high glucose DMEM supplemented with 10% (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamate (100 µg/mL) in a 37°C humidified atmosphere with 95% air and 5% CO2. Confluent macrophages (2 x 10^5 /well) were incubated with macrophage prompting lipopolysaccharide (LPS; 20 µg/mL; Sigma St. Louis, MO, USA) and were added at the same time with indomethacin (25-200 µg/mL) as the positive control (Ghimeray et al., 2015; Assanga et al., 2017; Arabiyat, et al., 2019) and the test compounds at different concentrations (5-200 µg/mL) for 24 hour incubations. A 100 µL Griess reagent (50 µL of 1% Sulfanilamide in 5% phosphoric acid and 50 µL of 0.1% napthylehtyllenediamine HCl) were mixed with aliquots of 100 µL of cell culture media and incubated at R.T. for 10 minutes. Absorbance at 550 nm was determined using a microplate reader (Biotek multiwell plate reader ELx800, USA). The concentration of nitrite was determined by comparing with the sodium nitrite standard curve. SRB cytotoxicity protocol was performed for the evaluation of the effect of studied compounds on RAW 264.7 viability (Huang et al., 2016; AbdulFattah et al., 2019).

DPPH Free Radical Scavenger Assay

This method depends on the reduction of the radicals resulting in a color change (from oxidized purple to reduced yellow). Mainly Diphenyl-2-picryl-hydrazyl (DPPH) undergoes reduction in a methanol (MeOH) solution in the presence of a hydrogen-donating compound due to the formation of the non-radical form DPPH-H. This change in color can be quantitatively measured using a spectrophotometer at 515–520 nm. In contrast to other radical scavenging assays, a DPPH radical is stable and can provide reproducible spectroscopic values (Sharma, and Bhat, 2009; Marinovaet al., 2011; Shalaby et al., 2013; Hidayat et al., 2-17; Haida, and Hakiman, 2019).. A DPPH solution (0.2 mM) was diluted with MeOH and then mixed with test compounds or ascorbic acid with a DPPH solution in a concentration ratio of 1:1 using a 96-well plate; the

treated solution was incubated one hour isolated from light. Finally, a change in absorbance at 517 nm wavelength was measured using a microplate reader (Bio-Tek Instrument, USA). Ascorbic acid was the robust and classical standard radical scavenging reference agent used for comparison purposes. The calculation of the DPPH radical scavenging activity inhibition was determined by the following equation, where A represents photometric absorbance: in% = (A control – A sample) / A control x 100% (Litwinienko et al., 2004; Sharma, and Bhat, 2009; Marinovaet al., 2011; Shalaby et al., 2013; Hidayat et al., 2-17; Haida, and Hakiman, 2019)

In Vitro Antiproliferative Assay

For cytotoxicity screening, MCF7 (ATCC® HTB-22) and T47D (ATCC® HTB-133) breast cancer cell lines, A375 human skin cancer cell line (ATCC® CRL-1619), HeLa human cervical cancer cell line (ATCC® CCL-2), PANC1 pancreatic cell line (ATCC® CRL-1469), A549 lung cancer cell line (ATCC® CCL-185), K562 leukemia cancer cell (ATCC® CCL-185) and HT29 colorectal cancer (CRC) cell line were procured (Kaur and Dufour, 2012; ATCC 2016). Periodontal ligament fibroblasts (PDL) were used for determinations of selective cytotoxicity. The cell lines were cultured in high glucose DMEM (Bio Whittaker, Verviers, Belgium) containing 10% FBS, HEPES Buffer (10 mM), L-glutamine (2 mM), gentamicin (50 µg/mL), penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL). The cells were incubated with the test compounds at different concentrations (5-200 µg/mL). The cytotoxicity measurements were determined using a Sulforhodamine B (SRB; Santa Cruz Biotechnology, Inc., Texas, USA) colorimetric assay for cytotoxicity screening (using a Spectro Scan 80D UV-VIS spectrophotometer, Sedico Ltd., Nicosia, Cyprus). The mechanism for the reduction of cell viability was adopted as described previously (Vichai and Kirtikara, 2006). As a robust and classical antineoplastic reference agent (El-Hamoly et al., 2017), cisplatin (1-200 µg/mL) was recruited for comparison purposes (Bacchi et al., 2012; Alabsi et al., 2018; Mamdooh et al., 2019; AlKhalil et al., 2020). Selectivity ratio (Selectivity index [SI]) is the term that describes the safety of the tested drugs. It was calculated by dividing the IC50 of the tested compounds on fibroblasts by the IC50 value of the same compound on any specific pathological cell line (Hoffman et al., 2011).

Statistical Analysis

The values were presented as mean ± SD of 3-4 independent experiments. The statistical differences between the reference agents and different treatment compounds were determined using the GraphPad Prism software unpaired t-test (version 5.01 for Windows; GraphPad software, San Diego, CA, USA). Values were considered significantly different if P < 0.05 and highly significantly different if P < 0.001.