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GREEN TEA EPIGALLOCATHECHIN-3-GALLATE (EGCG) - A NATURAL AGENT IN THE FIGHT AGAINST COLORECTAL CANCER

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Abstract

Green tea (GT) [*Camellia sinensis*] has since long been praised for its health benefits, ranging from protection against heart disease, neurodegenerative disease to cancer, among others. The Green Tea Polyphenols, strong antoxidants, of which (—)-epigallocathechin-3-gallate (EGCG) is the most abundant, are considered to be the active components responsible for its cancer prevention properties. Colorectal cancer (CRC) is the most frequent malignancy in the EU, being responsible for about 13% of all new cases of cancer. Out of all cases of gastrointestinal cancer, 50% are cases of CRC. Studies have suggested potential clinical activity of high doses of green tea for colorectal cancer prevention. However, there is much discrepancy between results of different studies and, thus, further testing is required. The aim of this project is to ascertain the cytotoxicity produced by GT-EGCG and its effectiveness in inhibiting the growth of a human colon cancer cell line (WiDr) versus a human normal colon cell line (CCD-841 CoN). Additive and synergistic effects of GT-EGCG when combined with conventional cancer therapies (FOLFIRI, BEVA-FOLFIRI, CAPOX, CETUXIMAB-FOLFIRI, FOLFOX, FU-FOL, CAPIRI and CAPOX) were also be studied using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) assay.

In this context an experimental study has been designed, divided in two main areas: *in vitro* studies with the WiDr cell line, and *in vivo* experiments with a subcutaneous tumour model in nude rats using the same cell line. Different EGCG enriched extracts have been used, comparing to EGCG pure for the in vitro experiments. An EGCG-enriched extract has been administered to the animals of the experiment. Results from both in vitro and in vivo studies are reported in this work, showing that EGCG may improve usual cancer treatment therapies.

Keywords: GREEN TEA, EGCG, COLORECTAL CANCER, WIDR, CHEMOTHERAPY

Resumo

O chá verde (*Camellia sinensis*) tem sido desde há muito elogiado pelos seus benefícios para a saúde, desde a proteção contra doenças cardíacas, doenças neurodegenerativas e ao cancro, entre outras. Os polifenóis do chá verde, antioxidantes fortes, dos quais o (—)-epigallocathechin-3-galato (EGCG) é o mais abundante, são considerados os componentes ativos responsáveis pelas propriedades de prevenção do cancro. O cancro colorectal (CCR) é a neoplasia maligna mais frequente na UE, sendo responsável por cerca de 13% de todos os novos casos de cancro. De todos os casos de cancro gastrointestinal, 50% são casos de CRC. Estudos têm sugerido potencial atividade clínica de altas doses de chá verde para a prevenção do cancro coloretal. No entanto, há muita discrepância entre os resultados de diferentes estudos e, portanto, mais testes são necessários. O objetivo deste projeto é verificar a citotoxicidade produzida pelo GT-EGCG e a sua eficácia na inibição do crescimento de uma linha celular de cancro do cólon humano (WiDr) *versus* uma linha celular de cólon normal humano (CCD-841 CoN). Uma possível sinergia entre GT-EGCG e regimes de quimioterapia contra o CRC (FOLFIRI, BEVA-FOLFIRI, CAPOX, CETUXIMAB-FOLFIRI, FOLFOX, FU-FOL, CAPIRI e CAPOX) foram estudadas, utilizando o ensaio de MTT.

Neste contexto, foi desenhado um estudo experimental, dividido em duas áreas principais: estudos *in vitro* com a linha celular WiDr e um modelo de tumor subcutâneo em ratos atímicos utilizando a mesma linha celular. Foram utilizados diferentes extratos enriquecidos em EGCG, comparando com EGCG puro para as experiências *in vitro*. Foi administrado um extrato enriquecido (em EGCG) aos animais da experiência. Os resultados dos estudos *in vitro* e *in vivo* são descritos neste trabalho, mostrando que o EGCG pode melhorar terapias convencionais no tratamento do cancro coloretal.

Palavras-chave: CHÁ VERDE, EGCG, CANCRO COLORECTAL, WIDR, QUIMIOTERPIA.

Abbreviations

ACRC	Colorectal cancer
BEVA	Bevacizumab
CA	p-coumaroylquinic acid
CAP	Capecitabine
CAPIRI	Capecitabine + irinotecan
CAPOX	Capecitabine + oxaliplatin
CCD-841 com	CCD 841 CoN (ATCC® CRL-1790™)
CET	Cetuximab
CET-FOLFIRI	Cetuximab + folinic acid + fluorouracil + irinotecan
C_f	Final concentratrion
CHUC	Coimbra Hospital and University Centre
C_i	Initial concentration
Conc	Concentration
CRC	Colorectal cancer
DMEM	Dulbecco's modified eagle media
dTMP	Thymidine monophosphate
EC	(—)-Epicatechin
EGC	(—)-Epigallocatechin
EGCG	(—)-Epigallocatechin gallate
EGCG-Pure	EGCG-Standard (minimum purity of 95%)
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine sérum
FDA	Food and Drug Administration
FFOL	Fluorouracil + folinic acid
FMUC	Faculty of Medicine of the University of Coimbra
FOL	Folinic acid
FOLFIRI	Irinotecan + fluorouracil + folinic acid
FOLFOX	FOL + OX
FU	Fluorouracil
G0	Control group
GA	Gallic acid
GC	(—)-gallocatechin
GCG	(—)-gallocatechin-3-gallate
GI	Chronic GTEE administration
GII	CRC without treatment
GIII	Protection + therapy
GIT	Gastrointestinal tract
GIV	Therapy
GT	Green tea
GTEE	Egcg extreme™
GTEP	Green tea extract powder

GTEs	GT extracts
GTPs	Green tea polyphenols
GV	Late therapy
H	Hour
HPLC	High performance liquid chromatography
H&E	Hematoxylin and eosin stain
IBILI	Institute for Biomedical Imaging and Life Sciences
IRI	Irinotecan
mCRC	Metastatic colorectal carcinoma
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Number of applications
OX	Oxaliplatin
PBS	Phosphate buffer saline
PDA	Photodiode-array detector
PI	Propidium iodide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
VGFR	Vascular endothelium growth factor receptor
WiDr	Widr (ATCC® CCL-218™)

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1. Introduction

Colorectal cancer (CRC) is currently the most frequent malignancy in the E.U., being responsible for about 13% of all new cases of cancer. In Portugal, there are about 5000 new cases every year, being the major cause of death by cancer. There has been a rise in the incidence of CRC in affluent countries, especially those where a low-to-high-income change took place. The same phenomena happened in Portugal, where the rates almost doubled since the 1970's. Such high increase in incidence points to a clear lifestyle effect upon the development of this cancer [1].

Although CRC can be hereditary, most cases (60-85%) are sporadic in origin [1]. Colorectal adenomas feature epithelial dysplasia and their size can range from mere 0.3 cm, frequently pedunculated lesions, to large, up to 10 cm, sessile polyps. While these are benign polyps and mostly do not progress to adenocarcinoma, they remain the source of the majority of colorectal adenocarcinomas. Besides adenomatous polyps, it should also be mentioned that cancer may arise form hyperplastic or form inflammatory polyps, although the likelihood of malignant transformation is extremely low [2].

Early diagnosis is one of the most important aspects to reduce CRC-associated mortality. The therapeutic modalities available to this tumour include surgery, chemotherapy and radiotherapy, as well as new antiangiogenic agents [1,3–5]. Patients with metastatic disease are candidates for systemic chemotherapy, aiming at systemic relief of symptoms and survival increase [1,3–6]. For many years, the first-line therapy for metastatic colorectal carcinoma (mCRC) was fluorouracil (FU), a thymidylate synthase inhibitor, which causes rapidly dividing cancerous cells to undergo cell death due to lack of thymidine monophosphate (dTMP). Current guidelines point to the use of an association of drugs as first line chemotherapy (Table 1 & 2) [3,4,7]. Namely, there are evidences that the combination of FU, folinic acid (FOL) and oxaliplatin (OX) or irinotecan (IRI) avails greater benefits [5]. The advances in the field of

molecular therapeutics led to the development of new weapons for the treatment of mCRC, devising monoclonal antibodies against the vascular endothelium growth factor receptor (VGFR) and epidermal growth factor receptor (EGFR) as their main targets [8]. The choice of which regimen to use is dependent on patient comorbidities, preferences regarding toxicities, as well as practical consideration, namely convenience and cost.

Table 1. Drug Combinations Used to Treat Colon Cancer. Adapted from [3].

Regimen Name	Drug Combination	Dose
AIO or German AIO	FOL, FU, and IRI	IRI (100 mg/m^2) and FOL (500 mg/m^2) administered as 2-hour infusions on d 1, followed by FU (2,000 mg/m^2) IV bolus administered via ambulatory pump weekly over 24 h, 4 times a y (52 wk).
CAPOX	CAP and OX	CAP (1,000 mg/m^2) bid on d 1–14, plus OX (70 mg/m^2) on d 1 and 8 every 3 wk.
Douillard	FOL, FU, and IRI	IRI (180 mg/m^2) administered as a 2-h infusion on d 1, FOL (200 mg/m^2) administered as a 2-h infusion on d 1 and 2, followed by a loading dose of FU (400 mg/m^2) IV bolus, then FU (600 mg/m^2) administered via ambulatory pump over 22 h every 2 wk on d 1 and 2.

FU = fluorouracil; AIO = Arbeitsgemeinschaft Internistische Onkologie; bid = twice a day; IFL = irinotecan, FU, and leucovorin; IV = intravenous; FOL = leucovorin; y = year; wk = week; d = day.

Table 1. Drug Combinations Used to Treat Colon Cancer (cont.). Adapted from [3].

Regimen Name	Drug Combination	Dose
FOLFIRI	FOL, FU, and IRI	IRI (180 mg/m^2) and FOL (400 mg/m^2) administered as 2-h infusions on d 1, followed by a loading dose of FU (400 mg/m^2) IV bolus administered on d 1, then FU (2,400–3,000 mg/m^2) administered via ambulatory pump over 46 h every 2 wk.
FOLFOX-4	OX, FOL, and FU	OX (85 mg/m2) administered as a 2-h infusion on d 1, FOL (200 mg/m^2) administered as a 2-h infusion on d 1 and 2, followed by a loading dose of FU (400 mg/m^2) IV bolus, then FU (600 mg/m^2) administered via ambulatory pump over 22 h every 2 wk on d 1 and 2.
FOLFOX-6	OX, FOL, and FU	OX (85–100 mg/m^2) and FOL (400 mg/m^2) administered as 2-h infusions on d 1, followed by a loading dose of FU (400 mg/m^2) IV bolus on d 1, then FU (2,400–3,000 mg/m^2) administered via ambulatory pump over 46 h every 2 wk.
FOLFOXIRI	IRI, OX, FOL, FU	IRI (165 mg/m^2) administered as a 60-min infusion, then concomitant infusion of oxaliplatin (85 mg/m^2) and FOL (200 mg/m^2) over 120 min, followed by FU (3,200 mg/m^2) administered as a 48-h continuous infusion.

Table 1. Drug Combinations Used to Treat Colon Cancer (cont.). Adapted from [3].

Regimen Name	Drug Combination	Dose
FUFOX	FU, FOL, and OX	OX (50 mg/m^2) plus FOL (500 mg/m^2) plus FU (2,000 mg/m^2) administered as a 22-h continuous infusion on d 1, 8, 22, and 29 every 36 d.
FUOX	FU plus OX	FU (2,250 mg/m^2) administered as a continuous infusion over 48 h on d 1, 8, 15, 22, 29, and 36 plus oxaliplatin (85 mg/m^2) on d 1, 15, and 29 every 6 wk.
IFL (or Saltz)	IRI, FU, and FOL	IRI (125 mg/m^2) plus FU (500 mg/m^2) IV bolus and FOL (20 mg/m^2) IV bolus administered weekly for 4 out of 6 wk.
CAPOX	CAP plus OX	Oral CAP (1,000 mg/m^2) administered bid for 14 d plus OX (130 mg/m^2) on d 1 every 3 wk.

For patients with a good performance status, the preferred initial therapy with bevacizumab (BEVA) is combined with one of the therapeutic regimens with most evidence support: OX with infusional FU and FOL (FOLFOX), irinotecan with infusional FU and FOL (FOLFIRI), or capecitabine (CAP) and OX (CAPOX). Data suggests that patients with a poor performance status benefit mostly from initial treatment with BEVA plus a fluoropyrimidine (FU or CAP) without a second cytotoxic agent [9].

Table 2. Drug Combinations Used to Treat Rectal Cancer. Adapted from [4].

Regimen Name	Drug Combination	Dose
FU = fluorouracil; AIO = Arbeitsgemeinschaft Internistische Onkologie; bid = twice a day; IFL = irinotecan, FU, and leucovorin; IV = intravenous; FOL = leucovorin; y = year; wk = week; d = day.		
AIO or German AIO	FOL, FU, and IRI	IRI (100 mg/m^2) and FOL (500 mg/m^2) administered as 2-h infusions on d 1, followed by FU (2,000 mg/m^2) IV bolus administered via ambulatory pump weekly over 24 h, 4 times a y (52 wk).
CAPOX	CAP and OX	CAP (1,000 mg/m^2) bid on d 1–14, plus OX (70 mg/m^2) on d 1 and 8 every 3 wk.
Douillard	FOL, FU, and IRI	IRI (180 mg/m^2) administered as a 2-h infusion on d 1, FOL (200 mg/m^2) administered as a 2-h infusion on d 1 and 2, followed by a loading dose of FU (400 mg/m^2) IV bolus, then FU (600 mg/m^2) administered via ambulatory pump over 22 h every 2 wk on d 1 and 2.
FOLFIRI	FOL, FU, and irinotecan	IRI (180 mg/m^2) and FOL (400 mg/m^2) administered as 2-h infusions on d1, followed by a loading dose of FU (400 mg/m^2) IV bolus administered on d 1, then FU (2,400–3,000 mg/m^2) administered via ambulatory pump over 46 h every 2 wk.

Table 2. Drug Combinations Used to Treat Rectal Cancer (cont.). Adapted from [4].

Regimen Name	Drug Combination	Dose
FOLFOX4	OX, FOL, and FU	OX (85 mg/m^2) administered as a 2-h infusion on day 1, FOL (200 mg/m^2) administered as a 2-h infusion on d 1 and 2, followed by a loading dose of FU (400 mg/m^2) IV bolus, then FU (600 mg/m^2) administered via ambulatory pump over 22 h every 2 wk on d 1 and 2.
FOLFOX6	OX, FOL, and FU	OX (85–100 mg/m^2) and FOL (400 mg/m^2) administered as 2-h infusions on d 1, followed by a loading dose of FU (400 mg/m^2) IV bolus on d 1, then FU (2,400–3,000 mg/m^2) administered via ambulatory pump over 46 h every 2 wk.
FOLFOXIRI	IRI, OX, FOL, FU	IRI (165 mg/m^2) administered as a 60-min infusion, then concomitant infusion of OX (85 mg/m^2) and FOL (200 mg/m^2) over 120 min, followed by FU (3,200 mg/m^2) administered as a 48-h continuous infusion.
FUFOX	FU, FOL, and OX	OX (50 mg/m^2) plus FOL (500 mg/m^2) plus FU (2,000 mg/m^2) administered as a 22 h continuous infusion on days 1, 8, 22, and 29, every 36 d.

Table 2. Drug Combinations Used to Treat Rectal Cancer (cont.). Adapted from [4].

Regimen Name	Drug Combination	Dose
FUOX	FU plus OX	FU (2,250 mg/m^2) administered as a continuous infusion over 48 h on d 1, 8, 15, 22, 29, and 36 plus OX (85 mg/m^2) on d 1, 15, and 29 every 6 wk.
IFL (or Saltz)	IRI, FU, and FOL	IRI (125 mg/m^2) plus FU (500 mg/m^2) IV bolus and FOL (20 mg/m^2) IV bolus administered weekly for 4 out of 6 wk.
CAPOX	CAP plus OX	Oral CAP (1,000 mg/m^2) administered bid for 14 d plus OX (130 mg/m^2) on d 1 every 3 wk.

Nevertheless, the treatment against cancer has been impaired by an increasing rate of chemo/radio-resistance, as well as recurrence of secondary tumours. Thus, major lifestyle changes are considered to be a crucial strategy, besides being cost-effective. In fact, decline in tobacco usage, fight against obesity and use of plant foods have already proven to be a realistic alternative.

The focus of many studies has been alternative agents, extracted from various plants, that are able to prevent or retard cancer initiation, promotion or progression and the aim of such studies has been to explore their efficacy and/or limitations in both experimental studies and clinical trials [10].

Green tea (GT) has since long been praised for its health benefits, ranging from protection against heart diseases, neurodegenerative diseases to cancer, among others [10]. This beverage

is an infusion made from the leaves of *Camellia sinensis* (Figure 1), a species of the Theaceae family [11].

Figure 1. *Camellia sinensis* [12].

The fresh leaves are steamed or fried at high temperatures, which inactivates the polyphenol oxidase, and stabilizes the monomeric catechins. This process prevents fermentation, which would yield oolong or black tea, depending on the extent, and produces a dried and stable product with a chemical composition similar to that of the fresh leaves [11,13–15]. The main constituents of the leaves are polyphenols (about 31-35%), methylxanthines (2 to 4%), represented mainly by caffeine (also known as tein), proteins (15%), hydrolysable tannins, soluble carbohydrates (about 5%), vitamins (B1, B2 and C) mineral nutrients (4 to 9%) and essential oil [11]. The Green Tea Polyphenols (GTPs), strong antioxidants, are considered to be the active components responsible for the cancer prevention properties of GT. The most abundant polyphenol constituents are gallic acid (GA), (—)-gallocatechin (GC), (—)-epicatechin (EC), (—)-epigallocatechin (EGC), (—)-epicatechin gallate (ECG), (—)-

epigallocatechin gallate (EGCG) (Figure 3), p-coumaroylquinic acid (CA), and (—)-gallocatechin-3-gallate (GCG). EC, ECG, EGC and EGCG are classified as flavonols, but commonly known as catechins [10,13]. These compounds, characterized by the feature of several phenol groups, aromatic rings with hydroxyl groups, are secondary metabolites. Their production ensures that the plant can adapt to hostile environment, pathogens, herbivores and competitors, by setting up an adequate protection against physical/chemical/biological damage. In term of reproductive advantages, they play a crucial role, attracting pollinators and seed disperses. In fact, these compounds are responsible for several specific flavors from our diet. Their unique physical, chemical and biological traits set them apart from other bioactive compounds. GTPs properties arise from featuring functional groups able to accept a free radical's negative charge and in sum delay/inhibit/prevent the oxidation of materials prone to oxidation by scavenging free radicals and decreasing the level of oxidative stress. It has been proven that polyphenols upregulate and aid the maintenance of an antioxidant defence, inhibit enzymes and chelate trace metals, suppress reactive oxygen species (ROS)/reactive nitrogen species (RNS) formation and scavenge radical species (RNS, ROS. $O2^{-}$, H_2O_2, OH^{*}, $ONOO^{-}$). It is thought that, *in vitro* they might, to some extent, inhibit the occurrence of the Fenton reaction [10].

Among the GTPs, EGCG is the one present in much higher concentrations in green tea. A cup of GT (200mL) may contain up to 100 mg of cathechins (Figure 2), of which 0.65mg (65%) consist of EGCG [10]. This work focuses on this flavonoid, EGCG, which belongs to the subclass of the flavanols/flavan-3-ols.

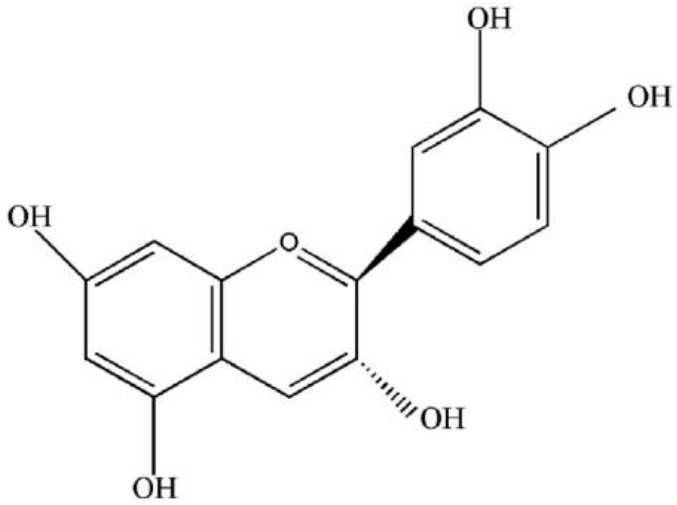


Figure 2. Chemical structure of catechins [10].

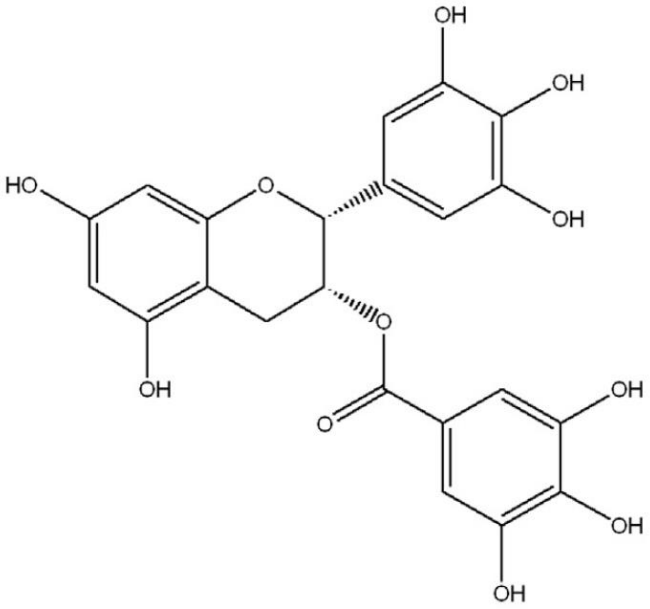


Figure 3. Chemical structure of (−)-epigallocatechin gallate (EGCG) [10]**.**

In vivo, EGCG's ability to act as competent bioactive molecule is limited by its bioavailability, i.e. on the extent of its biotransformation & conjugation upon absorption from the gastrointestinal tract (GIT), later in the liver and finally its uptake by cells. Most GTPs are absorbed in low amounts from the GI tract and reach the small intestine intact. Afterwards, polyphenols from the diet undergo extensive metabolization by glucosidase enzymes, phase I enzymes, such as cytochrome P450, which hydrolyze and oxidate, and also phase II enzymes, which are responsible for conjugation and detoxification. Mainly, these processes occur in the small intestine and liver. There are reports that only with oral doses of more than 1g of EGCG, were the maximal plasma concentrations higher than 1 μM. Administration of doses between 50 mg and 1,600 mg availed an EGCG concentration ranging from 130 to 392 ng/mL. Nonetheless, an increase in EGCG bioavailability was shown after chronic 800 mg administration [10,13]. On the other hand, some authors defend that EGCG may be metabolically activated to form more potent and effective bioactive compounds. It is also believed that ECGC may accumulate in tissues over time and, thus, reach cellular concentrations that are much higher than those which have been absorbed in clinical serum samples [13]. Several studies have shown that frequent green tea consumption will allow a maintenance of a high level of tea polyphenols. The organs which most likely benefit from the protective effects of these products are those who are most accessible to the polyphenols [10]. Biliary and urinary excretion are the main pathways of flavanols/flavan-3-ols excretion. Therefore, the intestine might actually be exposed to high levels of EGCG after ingestion, due to its biliary excretion.

Previous studies have shown that EGCG is capable of inducing cancer preventive metabolic changes, such as the enhancement of apoptosis, suppression of cell proliferation and inhibition of angiogenesis [16]. The anticancer activity of EGCG is thought to be bound to its antioxidant and pro-oxidant activity, and also to its ability to cause direct inhibition of certain molecular

targets [10]. GTPs seem to have antioxidant activity both in normal and cancer cells, protecting from oxidative damage. However, in cancer cells, which display a high level of ROS, the pro-oxidant effect of GTPs lead to a production of extra ROS, which destabilize the cancer cells leading to their death [10]. A study reported that EGCG's effect on cell proliferation appeared to be cancer cell-specific; their conclusion was that EGCG's ability to modulate gene expression would be different between normal cells and cancer cells [17]. EGCG used in combination with cancer therapeutic drugs has been shown to enhance their effects and counteract their toxicity, for example, doxorubicin and cisplatin, respectively [16–18]. The intestine seems to be a promising site for chemoprevention with polyphenols that have low systemic bioavailability after oral ingestion, namely EGCG. In fact, the absorbed EGCG is mostly excreted into the intestine through the bile. As such, the intestine/colon might actually be exposed to high levels of EGCG after ingestion.

1.1. Objectives

The fact is that it is still unclear whether the daily intake of GT, or of its isolated polyphenols, would affect the pharmacokinetics and pharmacodynamics of a chemotherapy regimen used by CRC patients [7,19]. It is, therefore, crucial to test the effects of such substances at a daily dose on the bioavailability and net therapeutic potential of co-administered drugs.

2. Materials & methods

2.1. Reagents and cell lines

The human cancer cell line (WiDr) and the normal human cell line (CCD-841 CoN) were kindly provided by the Biophysics Institute of the Faculty of Medicine of the University of Coimbra.

The drugs were kindly provided by the Coimbra Hospital and University Centre (CHUC) and Professor Rufino Silva (Espaço Médico de Coimbra). EGCG-Standard (minimum purity of 95%) was purchased from Sigma-Aldrich (EGCG-Pure) ([Prod. No. E4143]; EGCG Extreme™ (GTEE) was purchased from ProHealth, Inc., Green Tea Extract Powder (GTEP) was purchased from The Hut.com Ltd.; Lipton, Twinings, Taylors and Tetley Green Tea Lemon & Honey green teas were purchased from local markets. Dulbecco's Modified Eagle Media (DMEM), fœtal bovine serum (FBS), phosphate buffer saline (PBS), penicillin and streptomycin, trypsin, Annexin V and Propidium iodide (PI) kit for cell death evaluation, Propidium iodide (PI) kit for cell cycle studies, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA), following the established protocol in our laboratory [20–22]. All other materials were obtained from standard vendors.

2.2. High-performance liquid chromatography (HPLC) analysis of green teas and green tea extracts

EGCG is the most abundant polyphenol in GT and GTE (GT extract). Regardless, its concentration varies greatly between different GTE and its levels may be significantly different from the seller's original description. EGCG-Pure was used as a standard, in order to assess the EGCG content, to evaluate the quality of GTEs and to work as a standard for quality control. HPLC analysis was performed in a Gilson apparatus equipped with a photodiode-array detector (PDA). The studies were carried out on a spherisorb S5 ODS-2 column (250 x 4.6 mm i.d., 5 µm), Waters Corporation and a nucleosil guard cartridge C18 (30 x 4 mm i.d., 5 µm), Macherey-Nagel, at 24°C. A mobile phase, consisting of 5% aqueous formic acid v/v (A) and methanol (B), was used with a discontinuous gradient of 5–15% B (0–10 min), 15–25% B (10–15 min), 25-50% B (15–40 min), 50-80% B (40-50 min), followed by an isocratic elution during 10 min, at a flow rate of 1 mL/min. Chromatographic profiles were acquired in a 200–600 nm

wavelength range and were recorded at 280 and 320 nm. Data treatment was carried out with a dedicated software Unipoint® 2.10 Gilson [23].

2.3. Preparation of EGCG-enriched solutions

Solutions with the desired concentrations were obtained through sequential dilutions of GTEs' powder with sterile PBS, pH 7.4.

2.4. Preparation of green tea

One GT package was added into 200 mL of boiling water (95ºC). Water temperature was kept at 90º-95ºC and the tea package was steeped in hot water for 5 min while stirring. The beverage was let to cool down at room temperature in the dark, keeping the tea package inside. At the end of the process the volume of the final solution decreased to 180 mL, allowing some concentration.

2.5. Cell culture

The human CRC cell line WiDr and normal colon cell line CCD-841 CoN were cultured in DMEM culture medium supplemented with 10% FBS and 15% FBS, respectively, glutamine, and antibiotics (pen/strep 1%). Cells were kept in an incubator, at 37°C, with a 5% CO_2-humidified atmosphere. In order to transfer cells from flask to flask and microplates, trypsin 0.25% was used. Cells were centrifuged at 1,100 rotations per minute (rpm), for 5 min at 4ºC. For the experiments, these adherent cells were seeded onto 96-well microplates at 2.4×10^4 cells/well and cultivated for 48h. Six replicates were made per concentration, plus a six-sample control set per microplate. The cells were incubated with GTEs and GTs solutions with different

EGCG concentrations (0.01, 0.1, 0.5, 1, 5 and 10 µg/mL), which were added daily during the time of the experiment. All procedures were carried out in sterile conditions.

2.6. Study of cell viability after chronic treatment with EGCG-enriched solutions

The effect of repeated dose applications was evaluated at 24, 48, 72, 144, 216 and 288 h of incubation using GTEE. The same doses were reapplied every 24 h, i.e. 1, 2, 3, 6, 9 and 12 applications. The following concentrations: 0.01; 0.1; 0.5; 1; 5; 10 µg/mL were studied. Sextuplets of every sample were made plus a set of controls. For EGCG Pure, GTEP and GTs 3 and 6 repeated dose applications were evaluated, respectively at 72 and 144 h.

2.7. Study of cell viability after treatment with Food and Drug Administration (FDA) approved drugs / drug regimens for CRC

Several FDA approved drugs or drug regimens for CRC were studied: BEVA, CET, CAP, FU, IRI, FOL, OX, FOLFIRI, BEVA-FOLFIRI, CAPOX, CET-FOLFIRI, FOLFOX (FOL + OX), FFOL (FU + FOL). Concentrations were tested as indicated for human therapy, adjusted to the well surface area [24–26]. Drugs were kindly provided by CHUC.

2.8. Pharmacodynamics studies to assess how co-administration of EGCG-enriched solutions might impact the cytotoxicity of FDA approved drugs or drug regimens for CRC

Aforementioned drugs' administration was conducted according to the previously mentioned protocols and also exposed to different concentrations of GTEE. The same doses of GTEE were reapplied every 24h and the cytotoxicity was evaluated at 144 h.

2.9. Cell viability measurement

Cell viability and cytotoxicity were evaluated with the MTT assay, following treatment with EGCG-enriched solutions and/or, FDA approved drugs/drug regimens for CRC [20].

2.10. Flow cytometry

WiDr cells previously treated with aforementioned 6 days regimen of GTEE and/or BEVA-FOLFIRI were resuspended, divided into samples containing 5 x 10^5 cells and stained with Annexin V and PI kit, or PI kit, depending on the study. The number of fluorescent cells was quantified by flow cytometry (FACSCalibur, Becton Dickinson) [21,22].

2.11. Animals

26 male NIH-Foxn1rnu rats with 1.5 months of age were provided and housed at the Institute for Biomedical Imaging and Life Sciences / Faculty of Medicine of the University of Coimbra (IBILI/FMUC) animal facilities. These facilities are equipped according to the animal welfare legislation (cages and room with in/out controlled air, controlled humidity and temperature; maintained on 12h light/dark cycle). Food andwater/GTEE were available *ad libitum*. The animals were euthanized using a high carbon dioxide $c_1(CO_2)$ content gas. All efforts were made to minimise animal suffering[1]. The studies were performed according to the 2010/63/EU Directive on protection of animals used for scientific purposes [27].

[1] According to the Annex IV of the national law nº 113/2013, of August 7th regarding the severity of the experimental animal procedure [http://3dmfsx6ameqwfda31pu5rjxq.wpengine.netdna-cdn.com/wp-content/uploads/2015/10/Decreto-lei-113-12013-de-7-de-agosto.pdf].

2.12. Study protocol in rats

Rats were randomized into 6 groups: GI - chronic GTEE administration (n=3); GII - CRC without treatment (n=4); GIII - protection + therapy (n=4); GIV - therapy (n=3); GV - late therapy (n=4); G0 - respective controls (n=8). GI rats drank exclusively GTEE-containing solution for 5 months; GII rats were inoculate with WiDr cells, received no treatment and drank water; GIII rats were allowed to drink GTEE-containing solution, 2 months prior to tumour inoculation, and continued to drink GTEE solution for the remain of the experiment; GIV rats had only access to GTEE solution after being inoculated with WiDr cells; GV rats were allowed to drink only water for one month after tumour inoculation, after which, water was replaced by GTEE solution. Rats from all groups had ad libitum access to their respective drinking solutions. The solution had an EGCG concentration of 1625 mg/L; theoretically 5 times higher than that required to produce a plasma concentration of 0,1 μg/mL in a human [19].

2.13. Histological study of the organ samples

Histology is the study of cellular organization of body tissues and organs. The histologic technique is the procedure that has as its main goal to transform cells and tissues into preparations for light microscopy. The required steps occur in successive phases according to the fundamental principles of the histologic technique. These steps include fixation, dehydration, embedding in a suitable medium, sectioning into thin slices to enable the observation by transillumination. These slices were then processed for routine staining (Haematoxylin-Eosine) in order to obtain definitive preparations [28–30].

2.14. Statistical Analysis

Statistical analysis was performed using SPSS, version 23, and was analysed at a 5% significance level.

Group comparisons were performed using nonparametric tests due to usual sample sizes, namely Mann-Whitney test whenever there were two groups in analysis, or Kruskal-Wallis test with post-hoc tests, when justified, adjusted for multiple comparisons if there were three or more independent groups in analysis. Results were presented using mostly graphics, namely bar plots, boxplots or even graphs where each node represents a group and its mean rank.

Curve fit adjustment was performed in order to develop a predictive regression model to access the best dose-response curve explaining MTT using accumulated concentration over time, and results were graphically presented.

3. Results

3.1. EGCG content in green teas and green tea extracts

EGCG content in GTs (Table 3) and GTEs (Table 4) was determined by HPLC (High-performance liquid chromatography). Chromatographic profiles of the substances analysed are shown below (Figure 4-Figure 11).

Table 3. Quantitative assessment of EGCG infused during the preparation of GT from different brands.

Product	Tea bag content (g)	EGCG (mg) infused into tea (200 mL) per bag	EGCG (mg) infused into tea (200 mL) per gram of tea used
Lipton Pure Green Tea	1.4	46.06	32.9
Twinings Pure Green Tea	2	65	32.5
Taylors Pure Sencha Green Tea	1.5	43.5	29
Tetley Pure Green Tea	1.75	31.675	18.1

Table 4. EGCG Purity Assessment of GT from different brands.

Product	(%) EGCG
GTEE	54
GTE	7

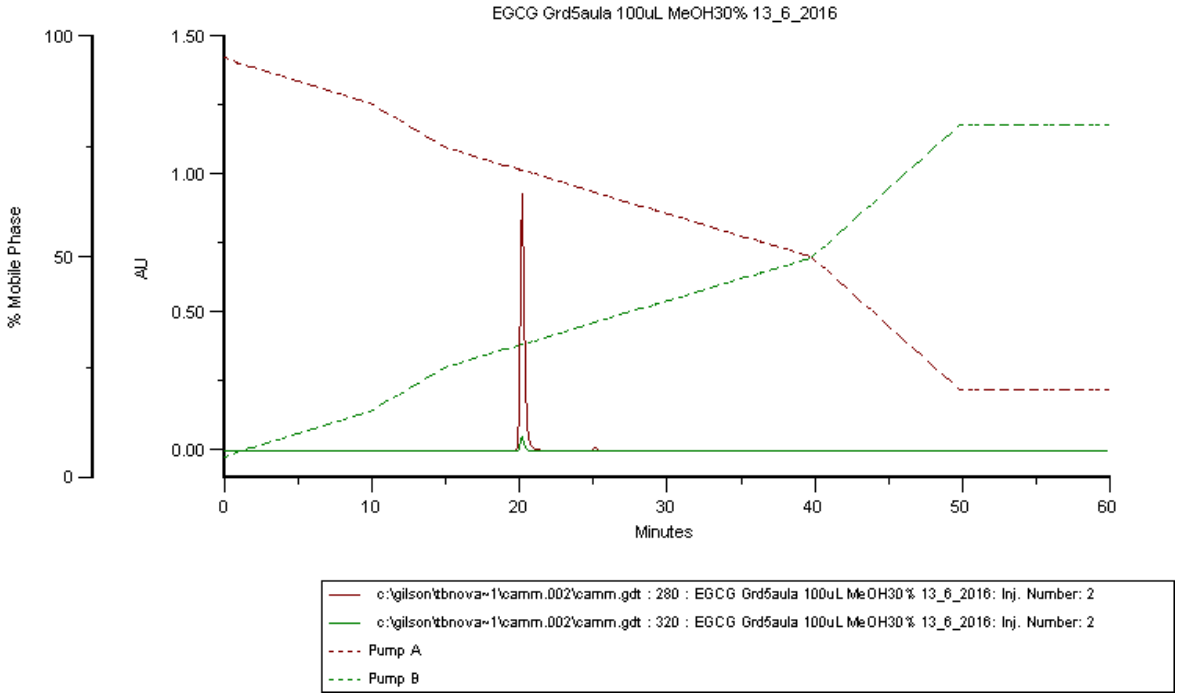


Figure 4. HPLC profile of EGCG-Pure obtained in the described conditions [23].

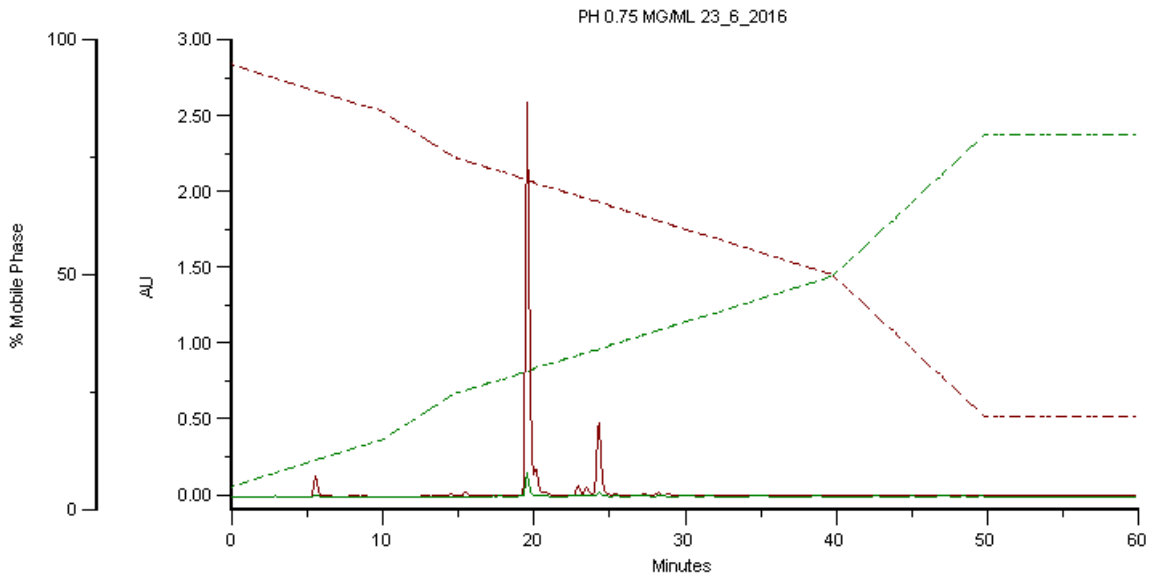


Figure 5. HPLC profile of GTEE obtained in the described conditions [23].

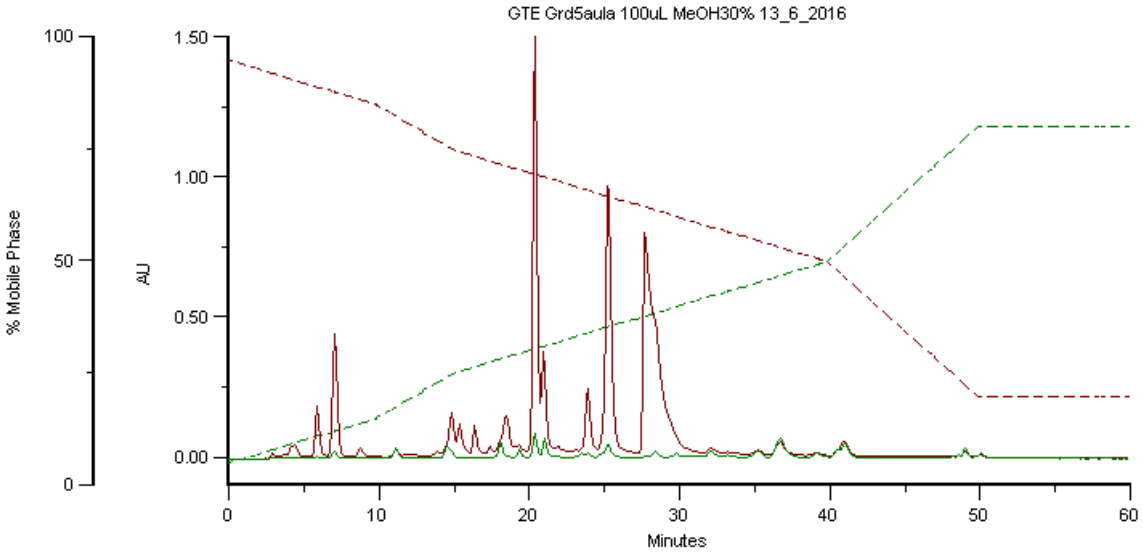


Figure 6. HPLC profile of GTEP obtained in the described conditions [23].

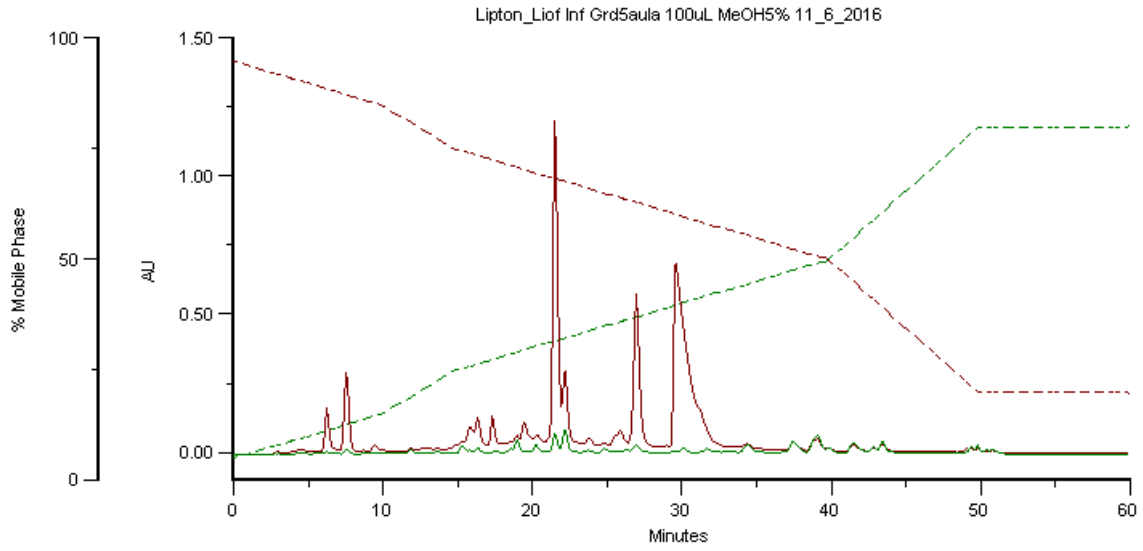


Figure 7. HPLC profile of Lipton tea obtained in the described conditions [23].

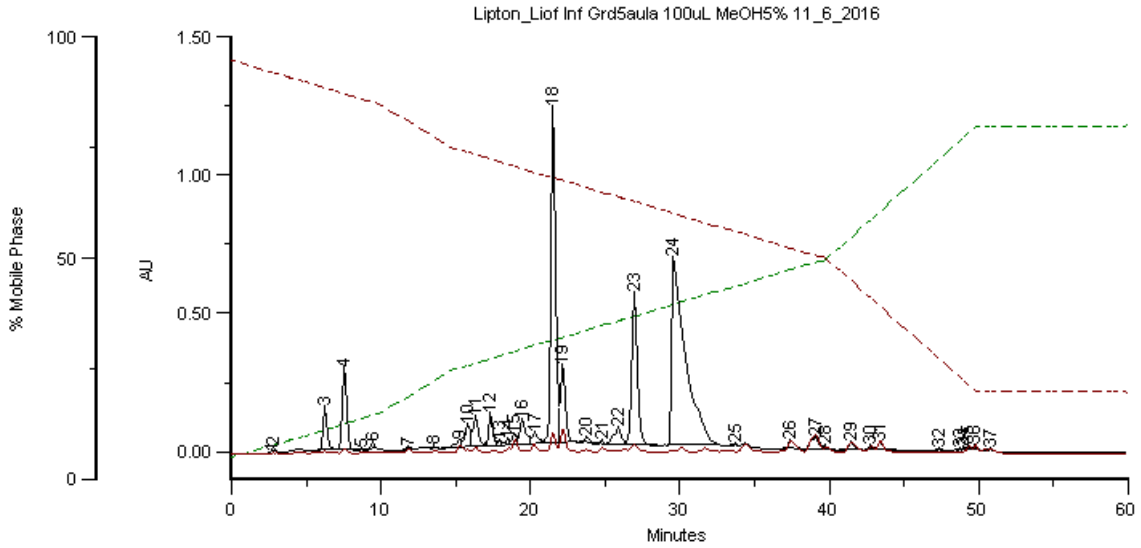


Figure 8. HPLC profile of Lipton tea obtained in the described conditions, showing the integration of the peaks [23].

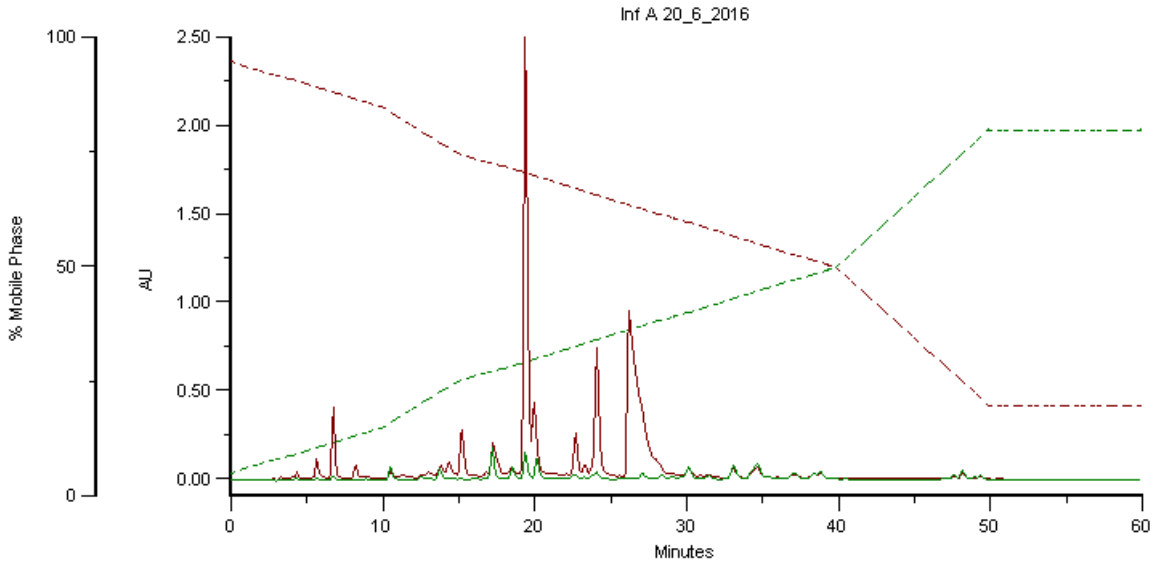


Figure 9. HPLC profile of Twinings tea obtained in the described conditions [23].

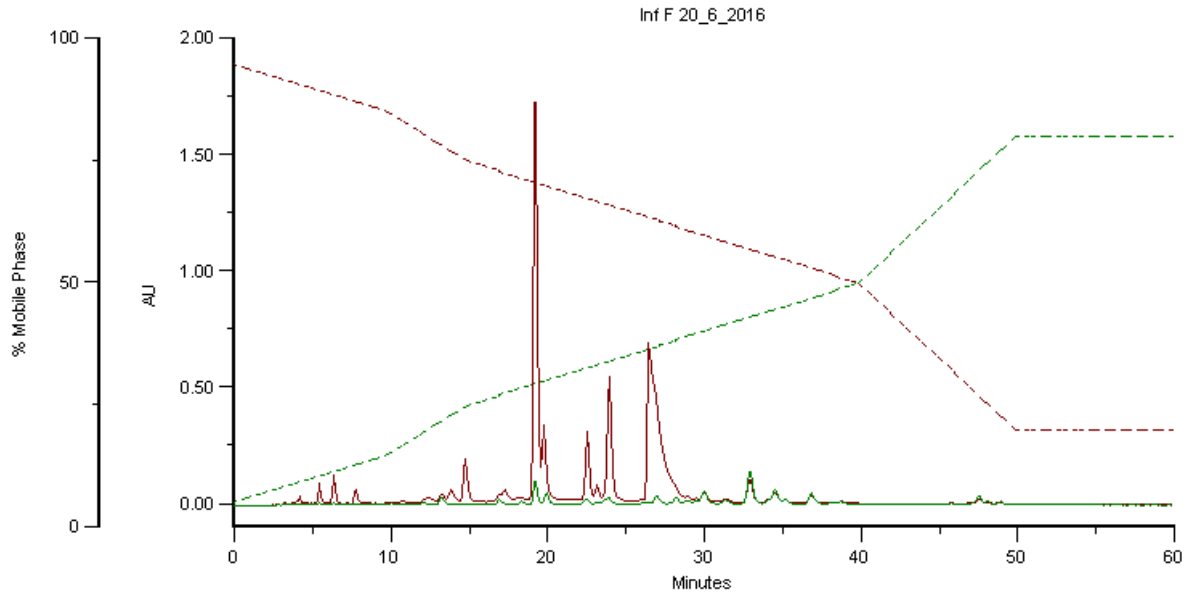


Figure 10. HPLC profile of Taylor's tea obtained in the described conditions [23].

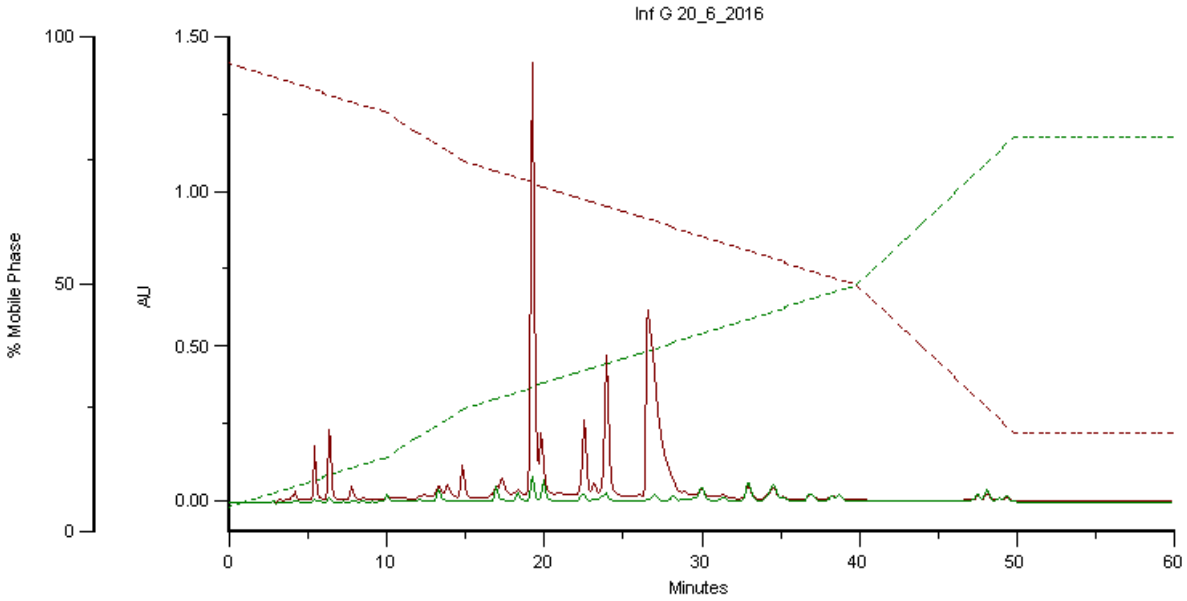


Figure 11. HPLC profile of Tetley Lemon & Honey tea obtained in the described conditions [23].

3.2. *In Vitro* studies

The concentrations of EGCG to be studied were chosen, considering reports on the actual absorption, as well as on the occurring likelihood of tissue concentration [19].

3.2.1. WiDr cell line viability after chronic exposure to GTs, and GTEs

The statistical models obtained were all well-adjusted (Figure 12). Neither the variable time, nor the EGCG concentration contribute in a statistically significant way. Only the interaction effect between EGCG concentration and time are statistically significant, except when stated otherwise.

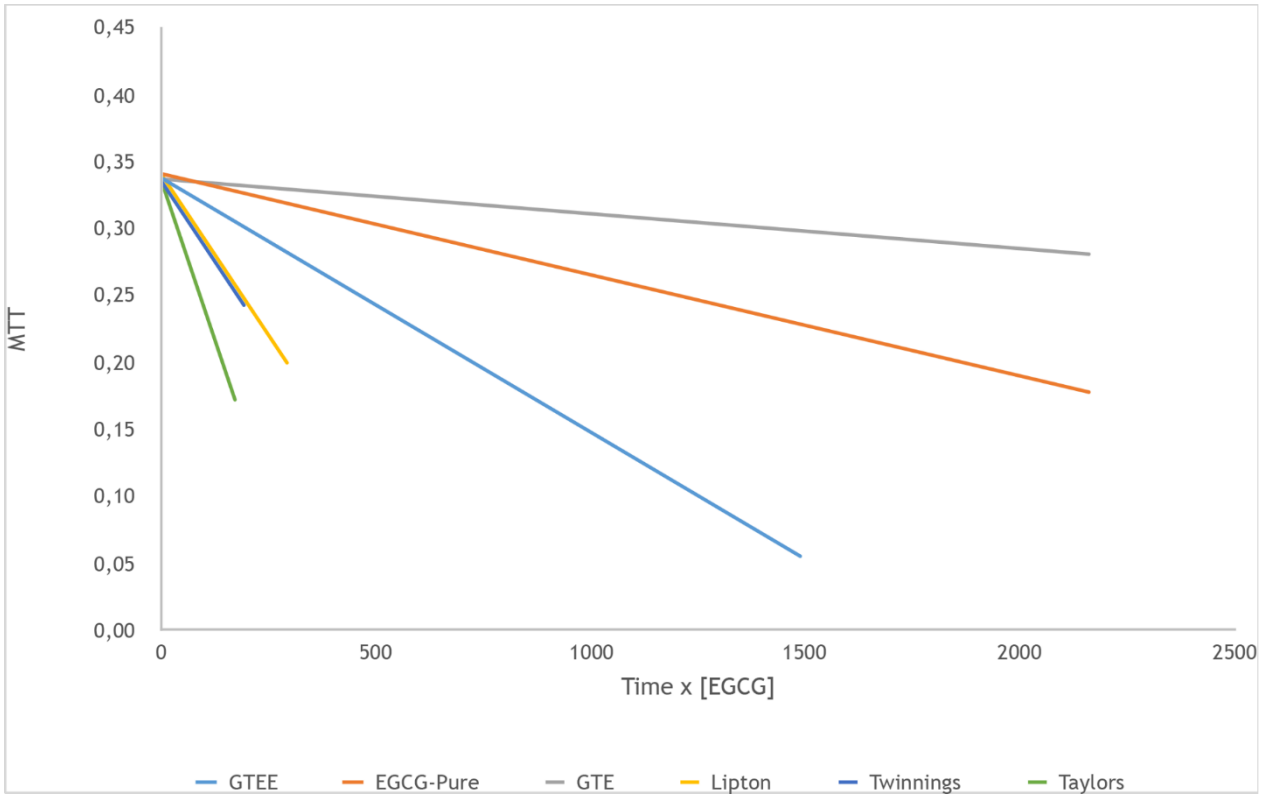


Figure 12. Median MTT observed values for WiDr colon cell line, according to exposure to EGCG concentration (µg/mL), over time, from GTEE, EGCG-Pure, GTE, Lipton, Twinings or Taylor's administration.

3.2.1.1. GT

3.2.1.1.1. Lipton

Neither the variable time, the EGCG concentration nor their interaction contribute in a statistically significant way to MTT variability. Regardless, when writing the model, considering the interaction between Time and GTE as the variable, it becomes statistically significant: MTT = 0.523 – 0.000248 x Time x LPT ($p < 0.001$, Figure 12).

LPT is statistically effective in reducing cell viability at 144 h ($p < 0.05$).

3.2.1.2. GTEs

3.2.1.2.1. EGCG-Pure

A well-adjusted model was obtained: MTT = 0.526 – 0.000227 x Time x [EGCG] ($p < 0.001$, Figure 12).

EGCG-Pure is statistically effective in reducing cell viability at 72 ($p < 0.05$) and 216 hours ($p < 0.001$).

3.2.1.2.2. GTEE

A predictive regression model with high correlation, for WiDr cell viability decrease (MTT values), using C_f (µg/mL) as independent variable, was obtained. C_f depends on the C_i (µg/mL) & GTEE NA (number of applications). Both model's significance and coefficient significance had a p value < 0.001 (Figure 13).

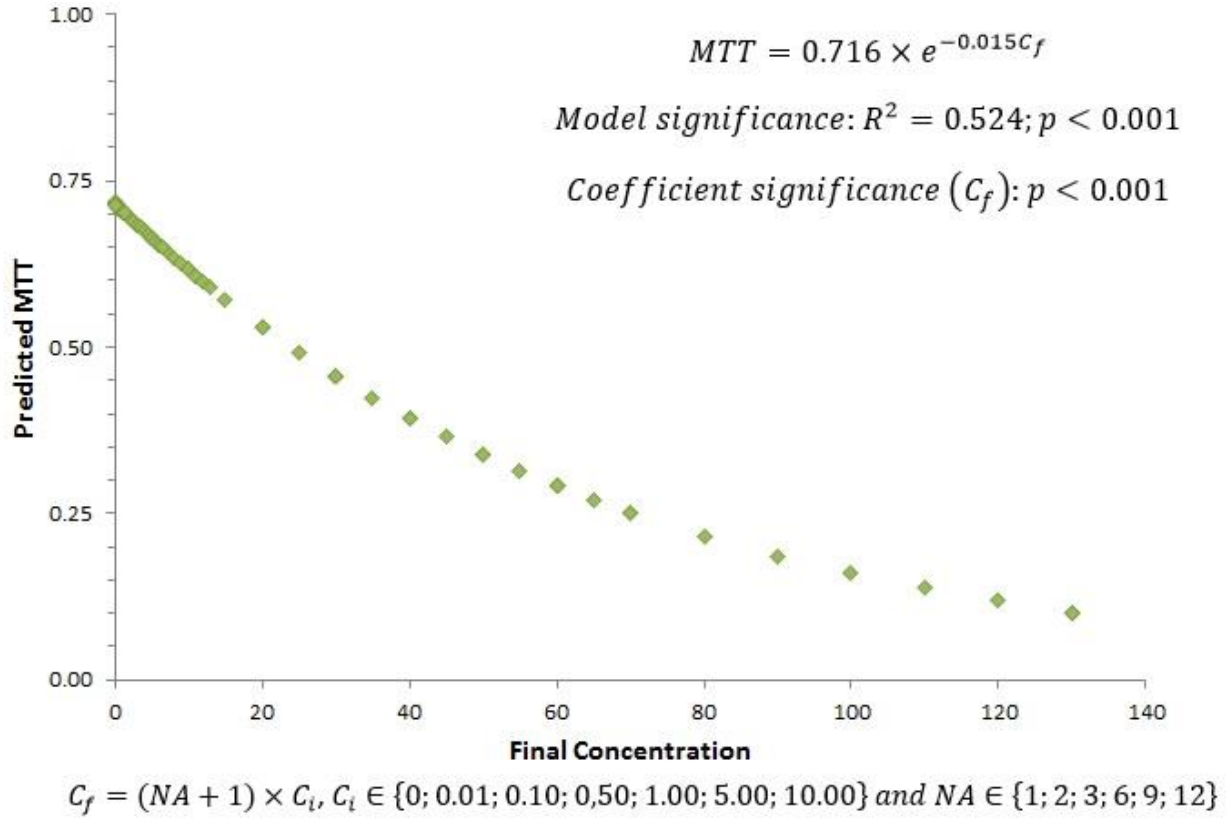


Figure 13. Predictive regression model for MTT values using C_f (µg/mL) as independent variable. C_f depends on the C_i (µg/mL) & EGCG NA.

All the concentrations tested decrease WiDr cells viability (Figure 13). In fact, the NA and concentrations explain 52.4% of found MTT variability. The weight of NA on MTT variation is higher than the weight of concentration, since the p value is more significant (for each extra application and increase to a greater concentration, MTT decreases respectively 0.113 and 0.008 units). For each extra unit on C_f (obtained either by the increase on C_i or on NA), there is a mean reduction of 8.55×10^3 units on MTT values.

The most WiDr-cytotoxic EGCG concentration is 10 µg/mL, being significantly effective with NA= 3 in decreasing WiDr cells viability ($p < 0.001$). The 5 µg/mL EGCG concentration is significantly effective after NA=6 ($p = 0.03$).

Furthermore, lower concentrations seem to achieve higher concentrations' cytotoxicity with the increase in the number of applications. These results mean that prolonged exposure to low doses EGCG, such as the EGCG bioavailability from GT consumption, might induce an equally effective WiDr cell-line cytotoxicity.

Another well-adjusted model was obtained: MTT= 0.531 - 0,000135 x Time x [EGCG] ($p<0.001$). GTEE is statistically effective in reducing cell viability at 72, 144, and 216 h ($p < 0.05$) (Figure 12).

3.2.1.2.3. GTEP

Neither the variable time, the EGCG concentration nor their interaction contribute in a statistically significant fashion to MTT variability. Regardless, when writing the model, considering the interaction between Time and GTEP as the variable, it becomes statistically significant: $MTT = 0.523 – 0.000179 \times Time \times GTEP$ ($p <0.001$) (Figure 12).

GTEP is statistically effective in reducing cell viability at 72, 144 ($p < 0.05$) and 216 h ($p < 0.001$).

3.2.2. Study of the cytotoxicity induced by co-administration of EGCG-enriched solutions and FDA approved drugs or drug regimens for Colorectal cancer

3.2.2.1. BEVA

Monotherapy with BEVA ($p = 0.024$), in the tested dosage (168 ng/well), does not induce a decrease of cell viability when compared with control ($p = 0.16$). Addition of GTEE ([EGCG] = 0.5 μg/mL), or GTEE ([EGCG] = 5 μg/mL) to the drug does not alter the cytotoxic effect of BEVA, since there is no statistically significant decrease of cell viability ($p = 0{,}103$, and $p = 1$, respectively) (Figure 14).

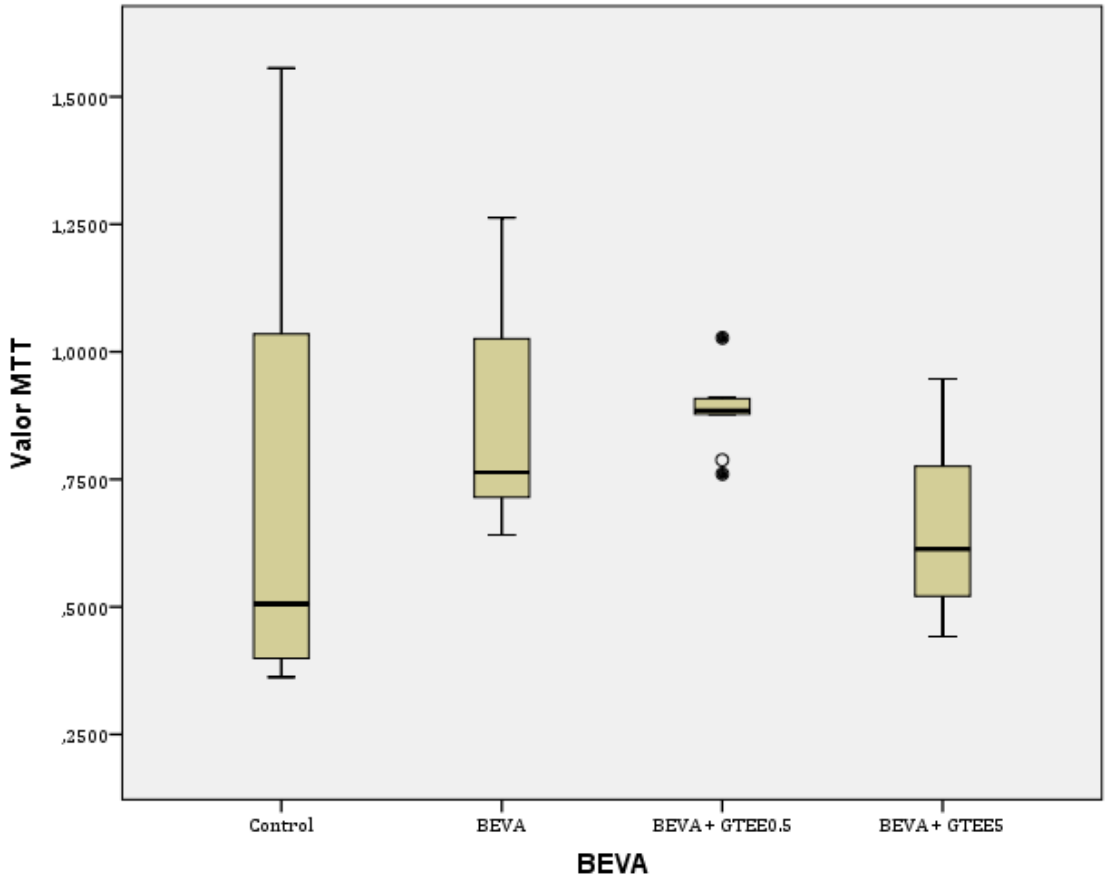


Figure 14. Boxplot for MTT distribution: controls, BEVA, BEVA + GTEE ([EGCG] = 0.5 μg/mL), and BEVA + GTEE ([EGCG] = 5 μg/mL.

3.2.2.2. CET

Monotherapy with CET ($p < 0.001$), for the tested dosage (112 ng/well), does not induce a decrease of cell viability when compared with control ($p = 1$). Addition of GTEE ([EGCG] = 0.5 μg/mL) or GTEE ([EGCG] = 5 μg/mL) to the drug statistically decreases cell viability in both cases ($p = 0.001$, and $p = 0.008$, respectively). (Figure 15).

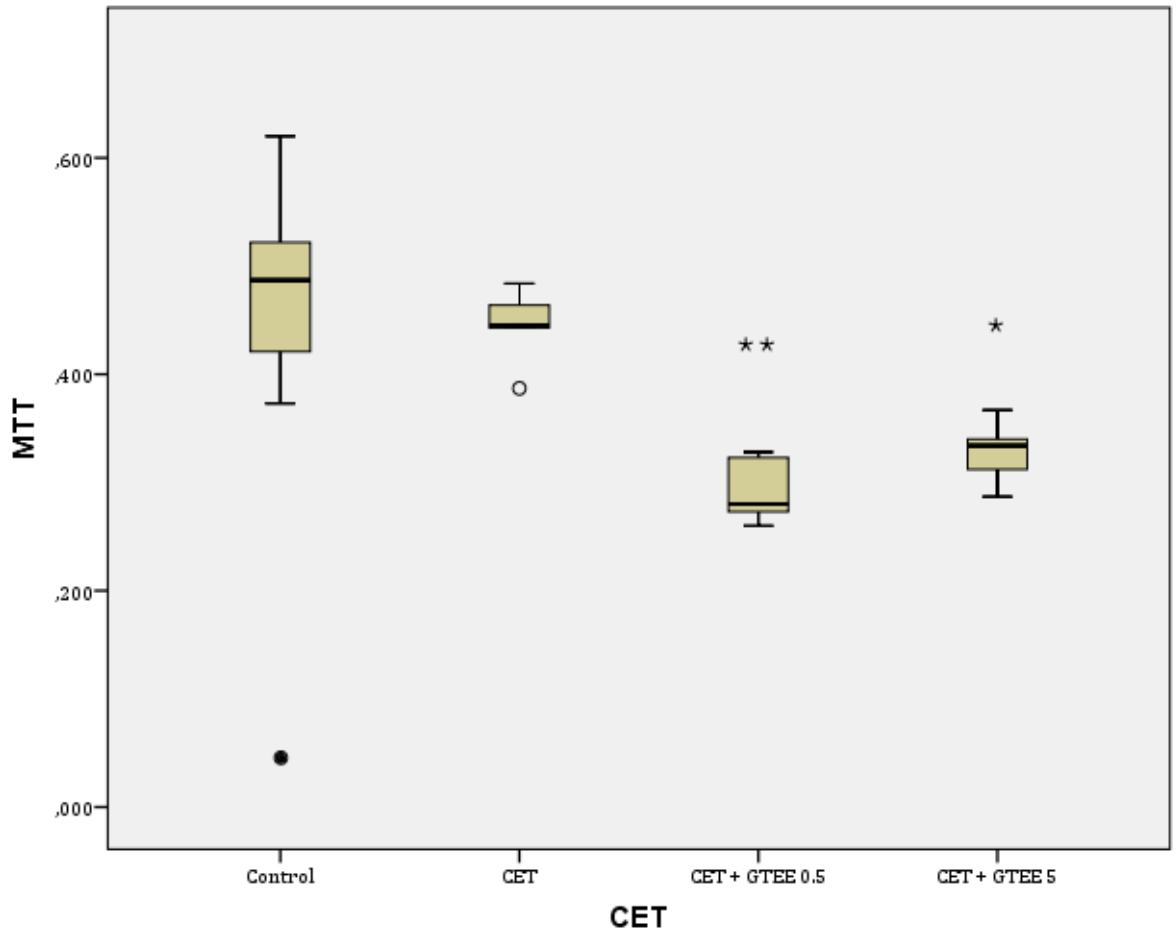


Figure 15. Boxplot for MTT distribution: controls, CET, CET + GTEE ([EGCG] = 0.5 μg/mL), and CET + GTEE ([EGCG] = 5 μg/mL.

3.2.2.3. CAP

Therapy with CAP ($p < 0.001$, 350 ng/well) does not induce decrease of cell viability when compared with control ($p = 0.368$), as well as GTEE ([EGCG] = 0.5 μg/mL) ($p = 1$). Adding GTEE ([EGCG] = 5 μg/mL) to CAP induces statistically significant decrease in cell viability ($p = 0.001$) (Figure 16).

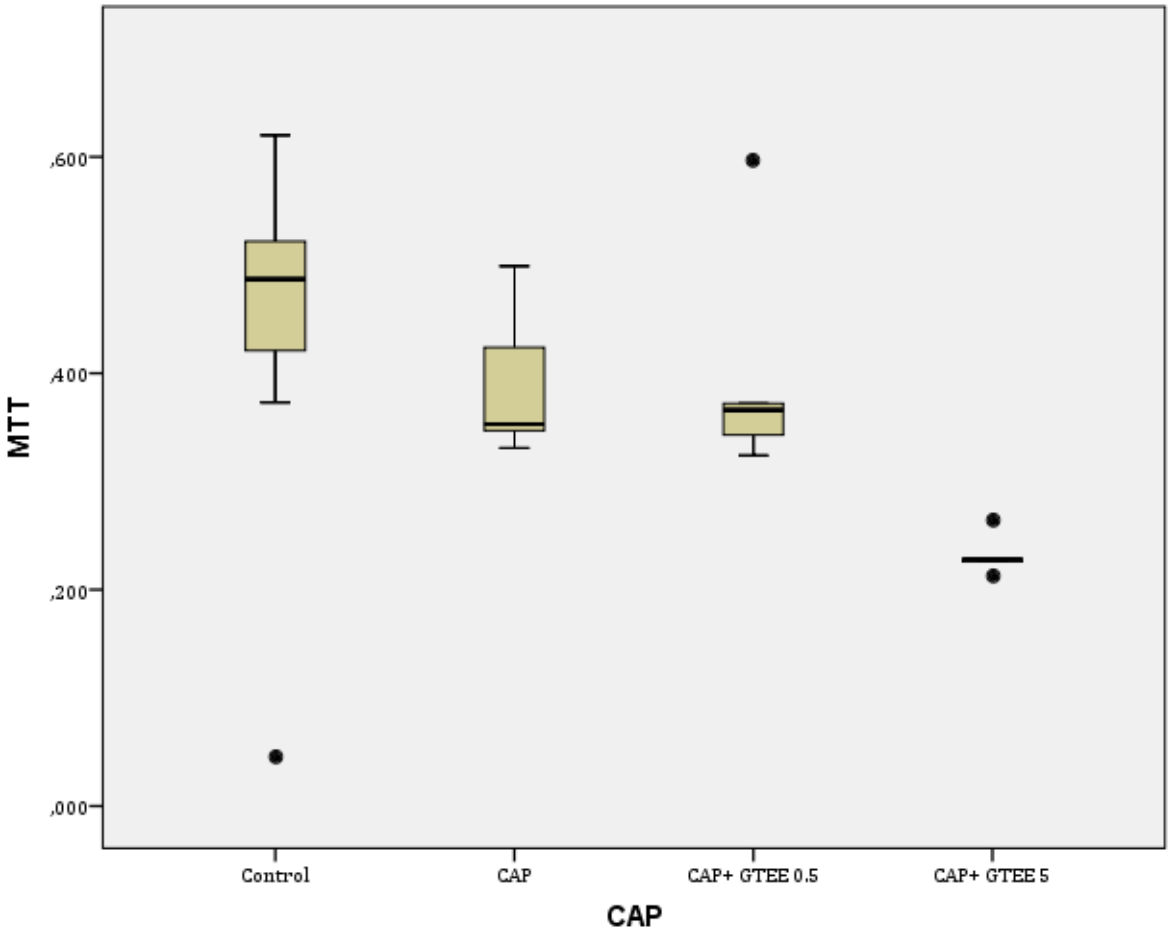


Figure 16. Boxplot for MTT distribution: controls, CAP, CAP + GTEE ([EGCG] = 0.5 μg/mL), and CAP + GTEE ([EGCG] = 5 μg/mL.

3.2.2.4. FU

Monotherapy with FU ($p < 0.001$, 112 ng/well) induces a significant statistical decrease when compared with control ($p < 0.001$), as well as FU + GTEE ([EGCG] 0.5 µg/mL) ($p < 0.001$), and FU + GTEE ([EGCG] 5 µg/mL) ($p < 0.001$) (Figure 17).

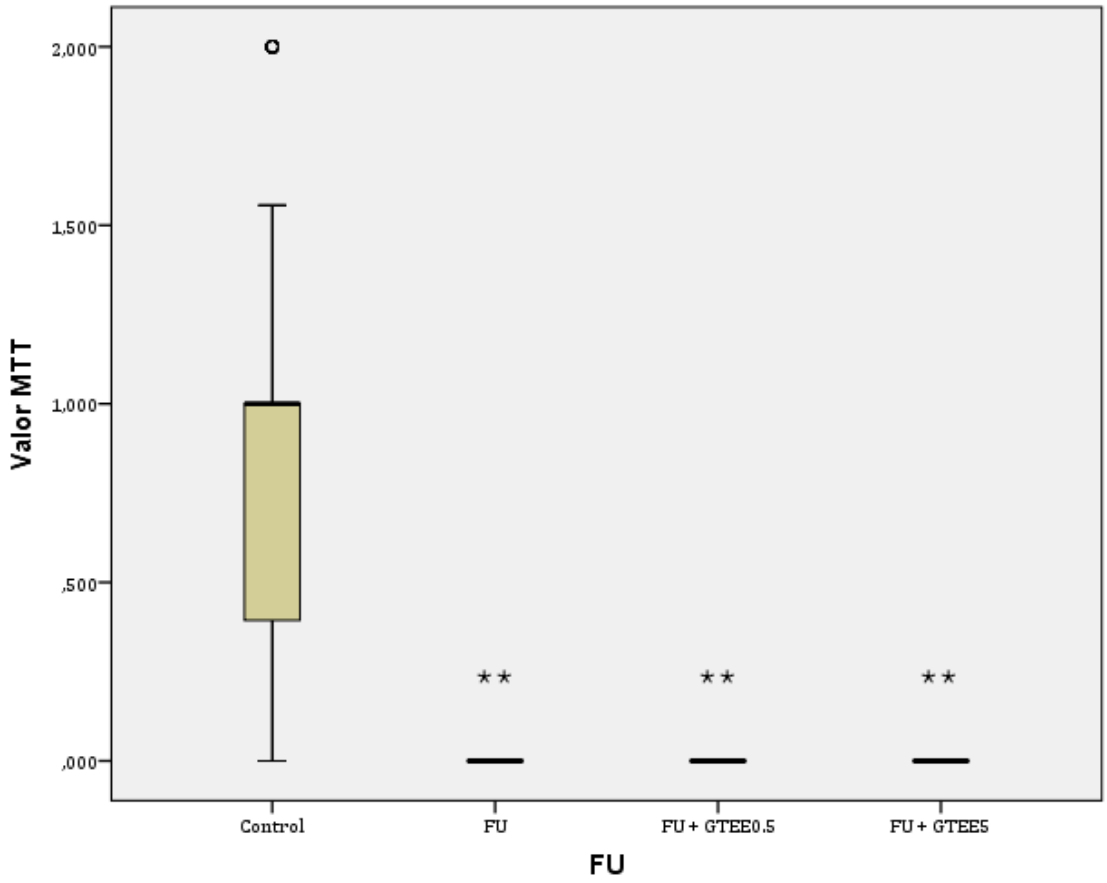


Figure 17. Boxplot for MTT distribution: controls, FU, FU + GTEE ([EGCG] = 0.5 µg/mL), and FU + GTEE ([EGCG] = 5 µg/mL.

3.2.2.5. IRI

Monotherapy with IRI ($p < 0.001$), in the tested dosage (98 ng/well), induces a significant statistical decrease when compared with control ($p< 0.001$), IRI + GTEE ([EGCG] 0.5 µg/mL) ($p< 0.001$), and IRI + GTEE ([EGCG] 5 µg/mL) ($p< 0.001$) (Figure 18).

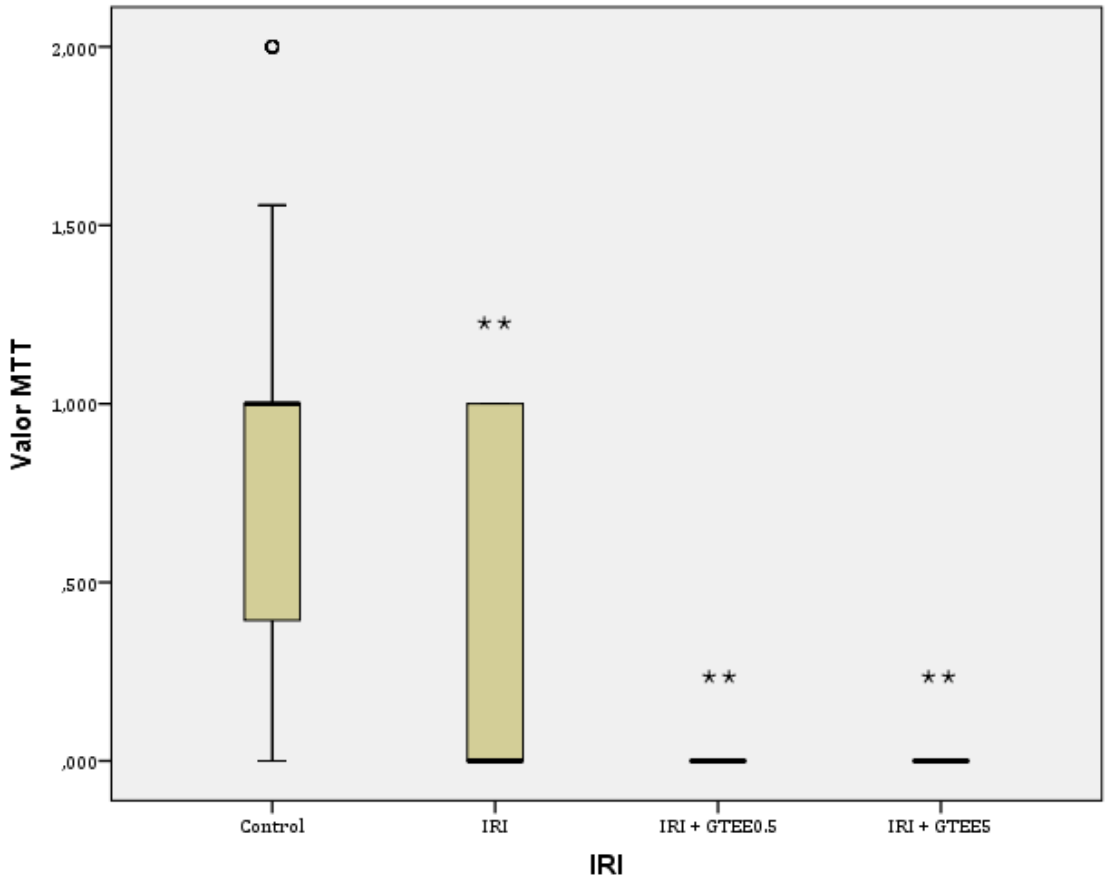


Figure 18. Boxplot for MTT distribution: controls, IRI, IRI + GTEE ([EGCG] = 0.5 µg/mL), and IRI + GTEE ([EGCG] = 5 µg/mL.

3.2.2.6. FOL

Monotherapy with FOL (p = 0.011, 168 ng/well) induces a significant statistically decrease in cell viability when compared with control (p< 0.05). Data analysis shows that addition of GTEE ([EGCG]= 0.5 µg/mL) or GTEE ([EGCG]= 5 µg/mL) do not induce an efficient decrease of cell viability (p = 1, and p = 1, respectively) (Figure 19).

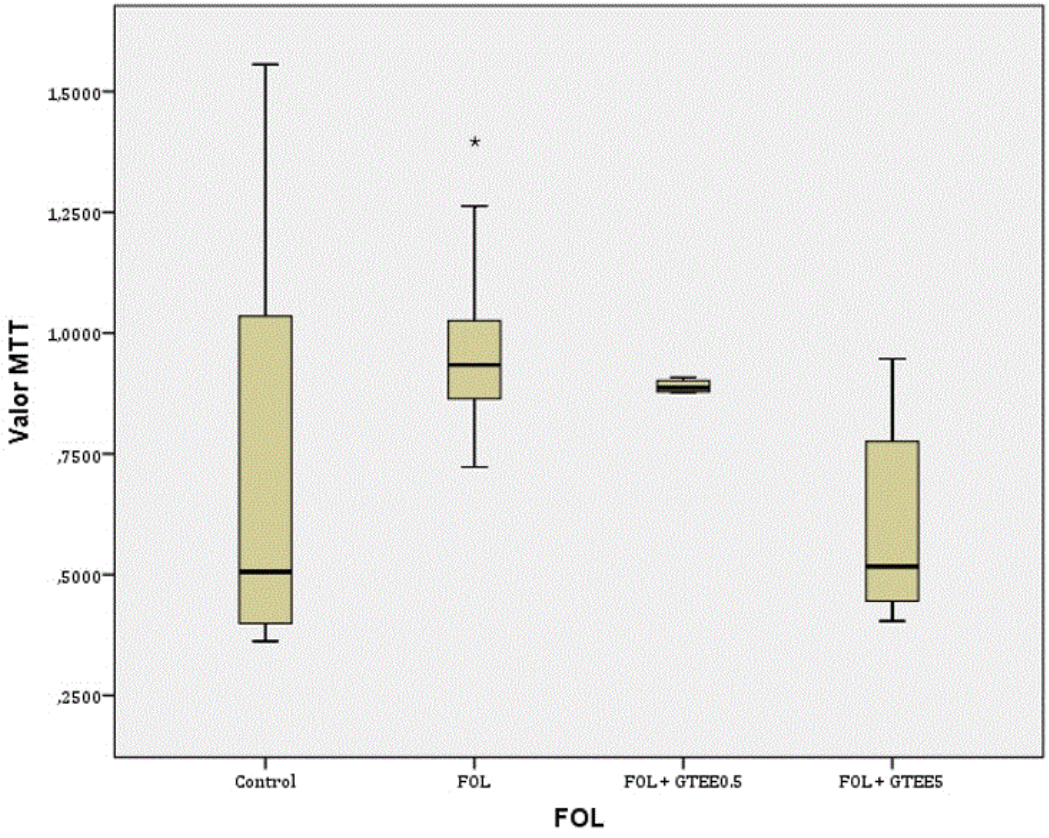


Figure 19. Boxplot for MTT distribution: controls, FOL, FOL + GTEE ([EGCG] = 0.5 µg/mL), and FOL + GTEE ([EGCG] = 5 µg/mL.

3.2.2.7. OX

Monotherapy with OX ($p < 0.001$), for the tested dosage (23.8 ng/well), does not induce a significant statistically decrease in cell viability when compared with control ($p = 0.814$). Addition of both GTEE ([EGCG] = 0.5 μg/mL) and GTEE ([EGCG] = 5 μg/mL) to OX induce a statistically significant decrease of cell viability ($p < 0.001$, and $p < 0.05$, respectively) (Figure 20).

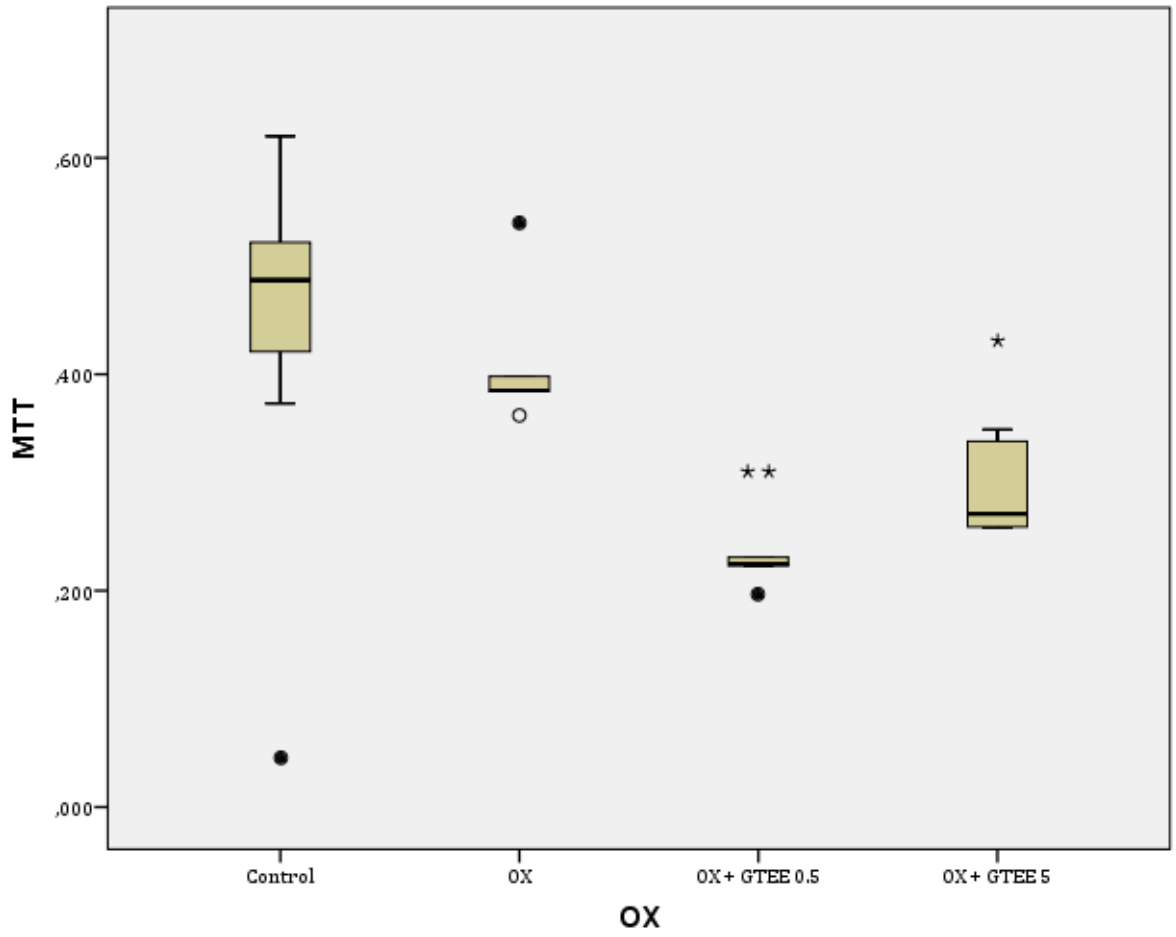


Figure 20. Boxplot for MTT distribution: controls, OX, OX + GTEE ([EGCG] = 0.5 μg/mL), and OX + GTEE ([EGCG] = 5 μg/mL.

3.2.2.8. CAPOX

CAPOX alone is not statistically effective (p= 0.092), becoming significant when GTEE ([EGCG] = 0.5 μg/mL) (p = 0.011) or GTEE ([EGCG] = 5 μg/mL) (p< 0.001) are added; there is no statistical significant difference between CAPOX and CAPOX + GTEE ([EGCG] = 0.5 μg/mL) (p= 0.392), but the conjugation with GTEE ([EGCG] = 5 μg/mL) is statistically significative (p= 0.035) (Figure 21).

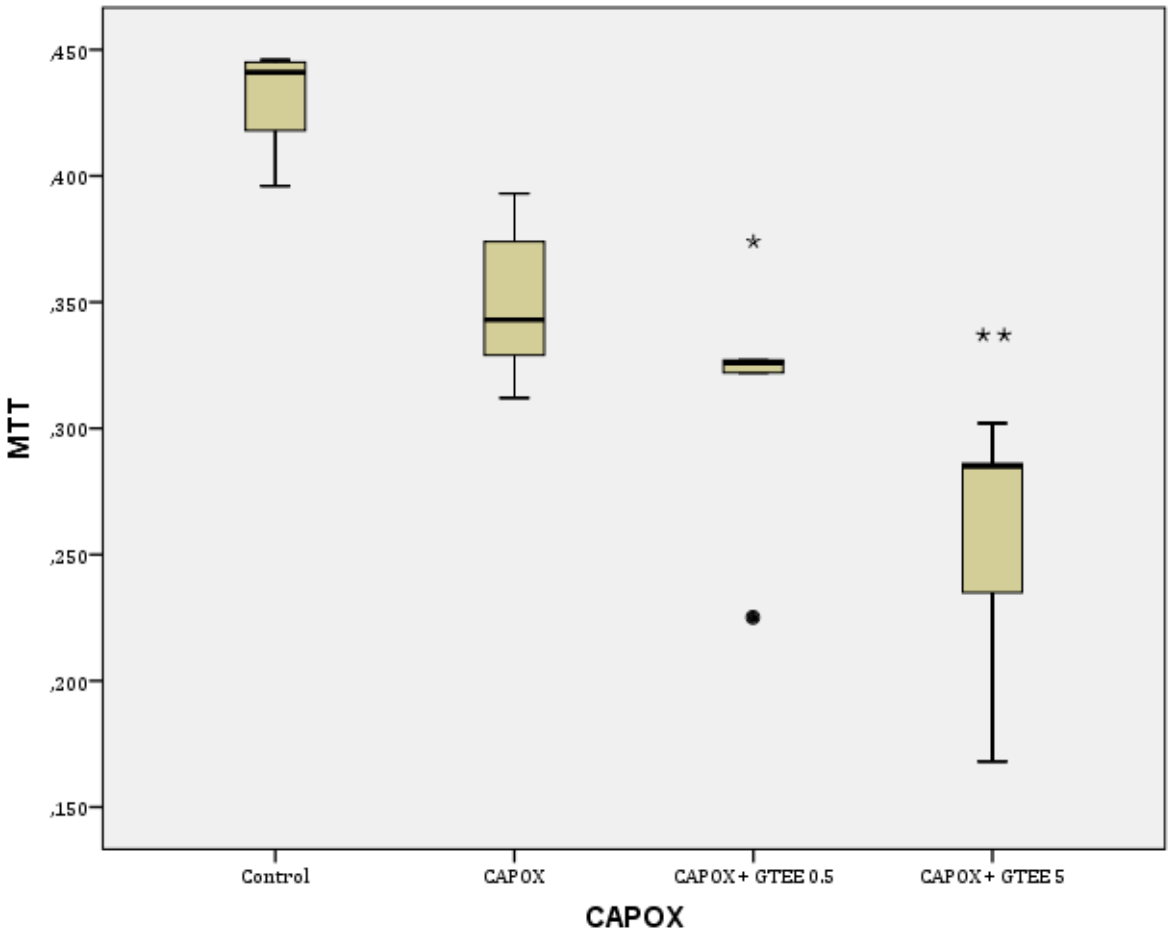


Figure 21. Boxplot for MTT distribution: controls, CAPOX, CAPOX + GTEE ([EGCG] = 0.5 μg/mL), and CAPOX + GTEE ([EGCG] = 5 μg/mL.

3.2.2.9. CET-FOLFIRI

CET-FOLFIRI therapy ($p = 0.025$), for the tested dosage (CET= 112 ng/well, FOL= 112 ng/well, FU= 112 ng/well, and IRI= 50.4 ng/well), does not induce a significant statistical decrease of cell viability when compared to control. Addition of GTEE ([EGCG] = 0.5 μg/mL) to CET-FOLFIRI induces a significant decrease of cell viability ($p< 0.05$), but adding GTEE ([EGCG] = 5 μg/mL) to CET-FOLFIRI does not induce a significant cell viability decrease (Figure 22).

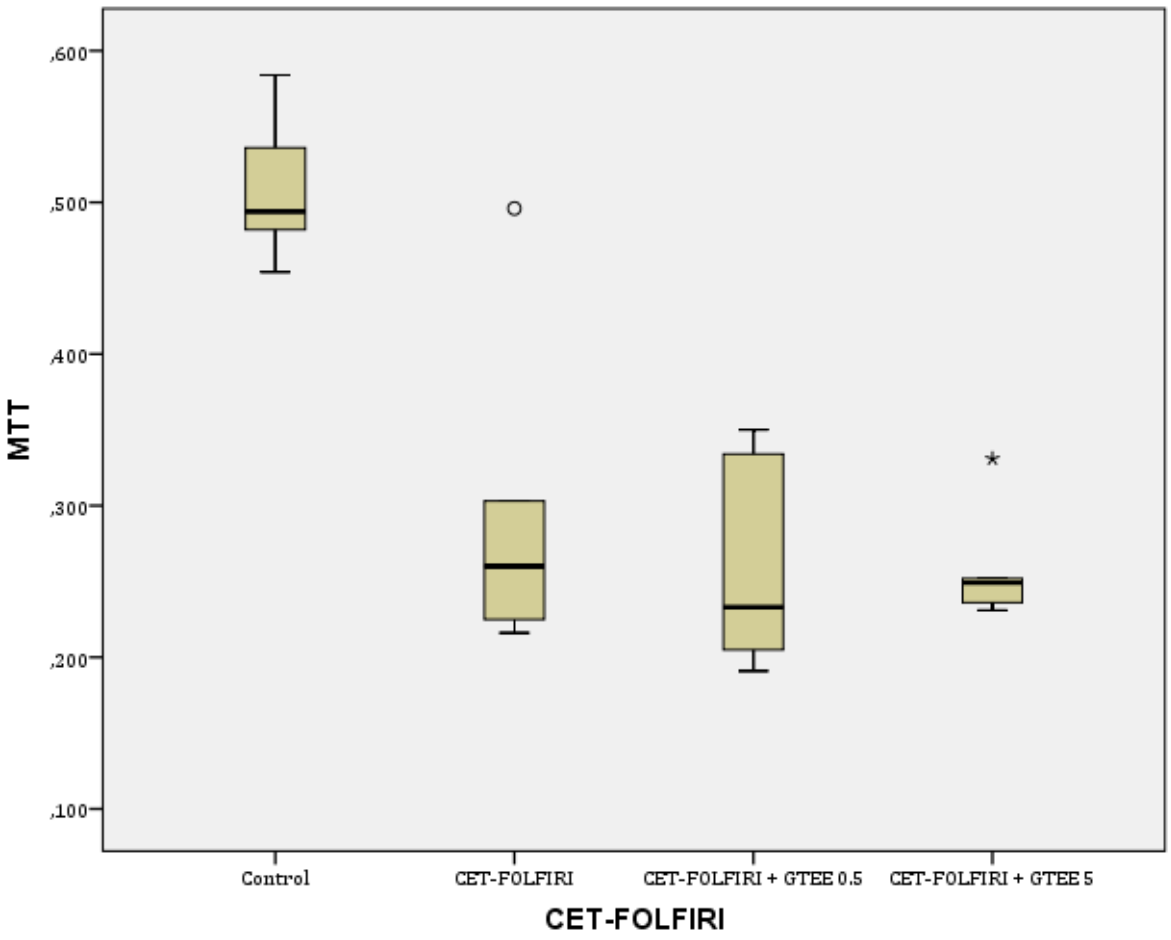


Figure 22. Boxplot for MTT distribution: controls, CET-FOLFIRI, CET-FOLFIRI + GTEE ([EGCG] = 0.5 μg/mL), and CET-FOLFIRI + GTEE ([EGCG] = 5 μg/mL.

3.2.2.10. FOLFOX

Combined therapy using FOLFOX (p= 0.001), for the tested dosage, does not significantly decrease cell viability when compared to control. Addition of GTEE ([EGCG] = 0.5 μg/mL) or GTEE ([EGCG] = 5 μg/mL) to FOLFOX induces a significant cell viability decrease in both cases (p< 0.05) (Figure 23).

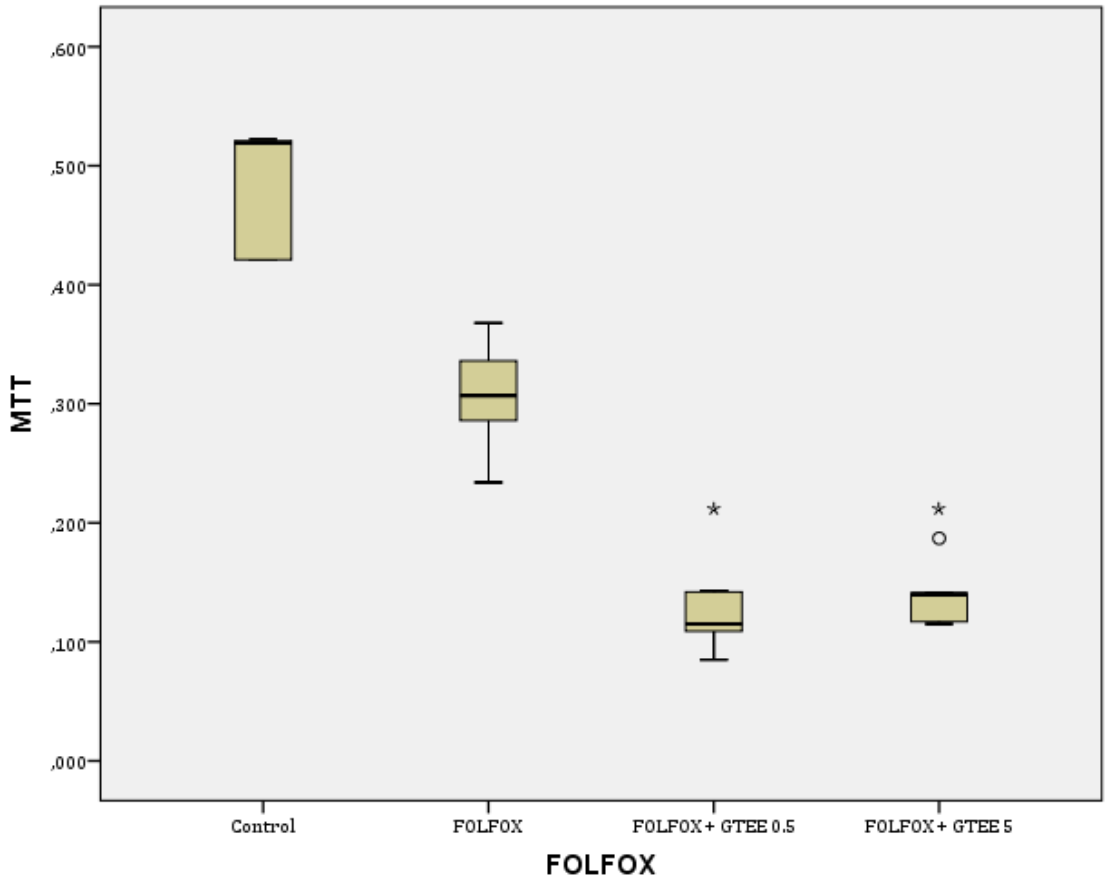


Figure 23. Boxplot for MTT distribution: controls, FOLFOX, FOLFOX + GTEE ([EGCG] = 0,5 μg/mL), and FOLFOX + GTEE ([EGCG] = 5 μg/mL.

3.2.2.11. CAPIRI

CAPIRI ($p = 0.002$), for the tested dosage, induce a decrease of cell viability when compared to control, although it is not statistically significant. Addition of GTEE ([EGCG] = 0.5 μg/mL) or GTEE ([EGCG] = 5 μg/mL) to CAPIRI induces a significant decrease of cell viability in both cases ($p< 0.05$) (Figure 24).

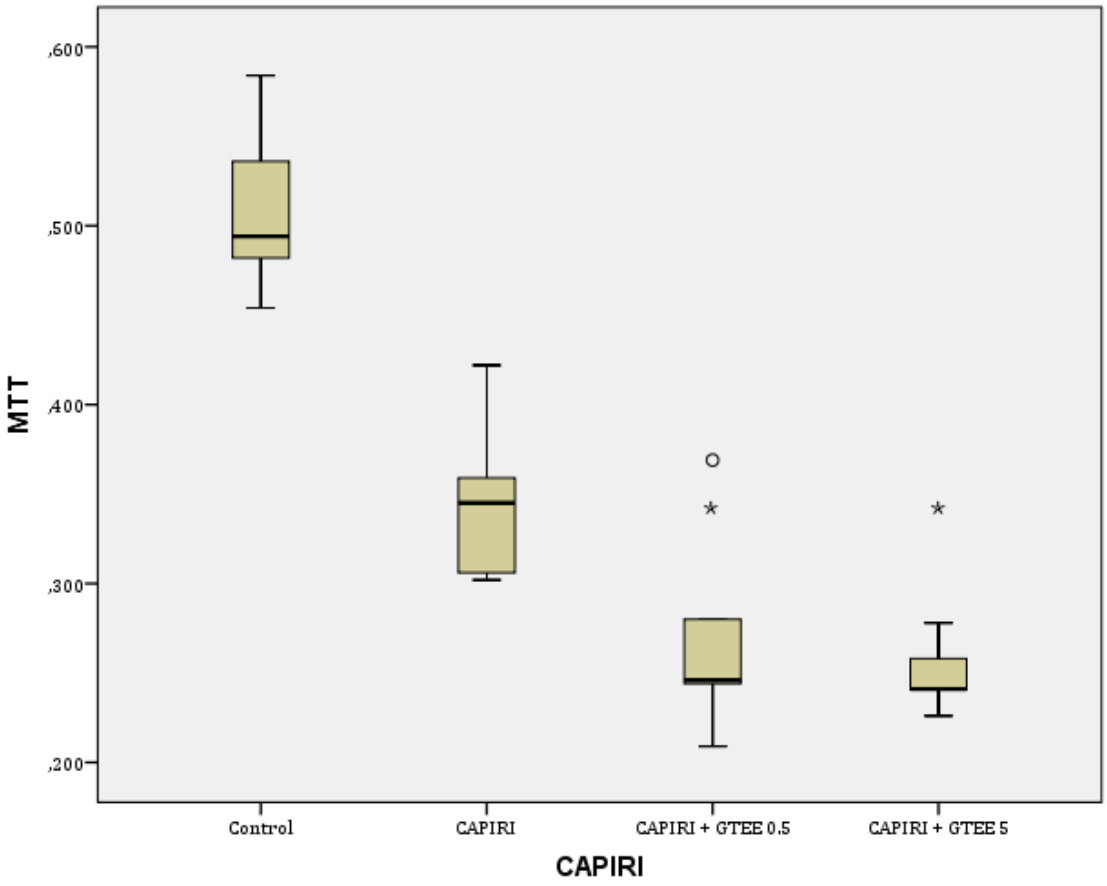


Figure 24. Boxplot for MTT distribution: controls, CAPIRI, CAPIRI + GTEE ([EGCG] = 0,5 μg/mL), and CAPIRI + GTEE ([EGCG] = 5 μg/mL.

3.2.2.12. CAPOX

CAPOX (p = 0.001) and CAPOX + GTEE ([EGCG] = 0.5 μg/mL) therapies induce a decrease of cell viability when compared to control, although not being statistically significant. However, CAPOX + GTEE ([EGCG] = 5 μg/mL) induces a statistically significant reduction of cell viability (p< 0.05) (Figure 25).

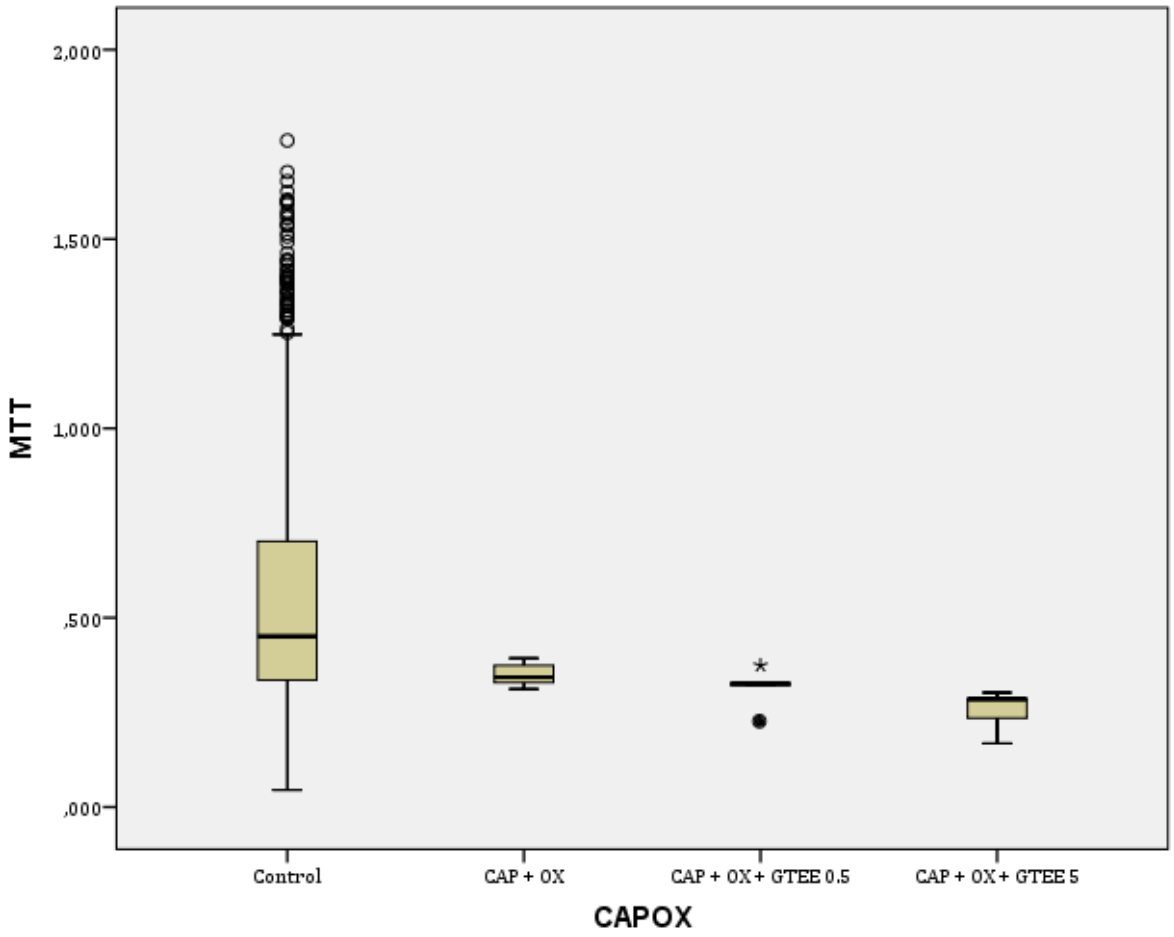


Figure 25. Boxplot for MTT distribution: controls, CAPOX, CAPOX + GTEE ([EGCG] = 0.5 μg/mL), and CAPOX + GTEE ([EGCG] = 5 μg/mL.

3.2.3. CCD-841CoN cell line viability after chronic exposure to GTEE

Cellular Viability of CCD-841 CoN shows no statistically significant difference between groups (Kruskal-Wallis test: p = 0.095) (Figure 26).

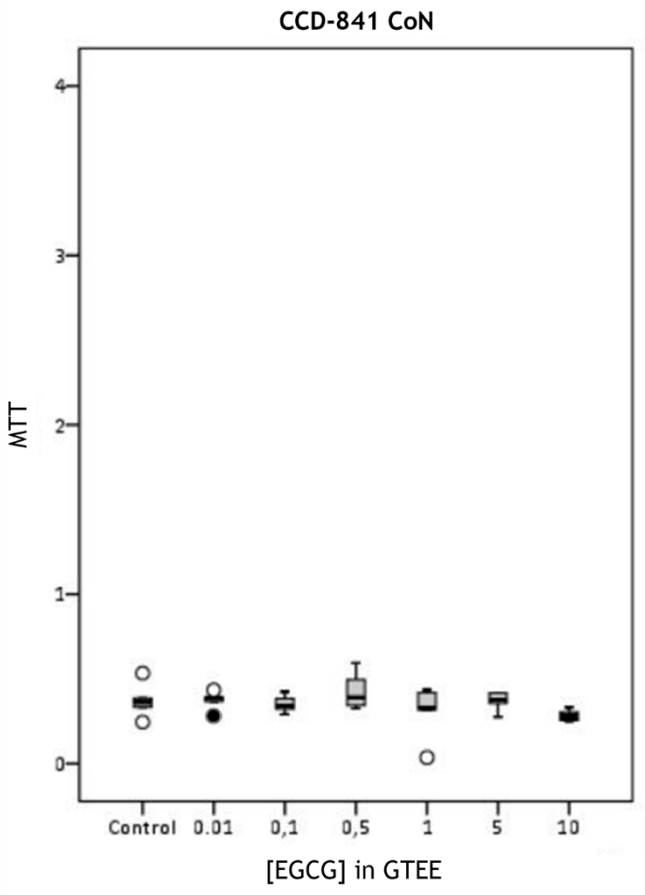


Figure 26. Median MTT observed values for Normal (CCD-841 CoN) colon cell line, according to exposure to EGCG concentration (µg/mL) from GTEE administration.

3.2.4. Flow cytometry

After asserting that GTEE selectively kills the cancer cells, we have sought to determine what type of cell death is induced. For this purpose, flow cytometry was performed, using different markers (kit of Annexin V+ Propidium Iodide, as previously referred) [21,22].

Firstly, the impact of different GTEE concentrations ([EGCG]= 5 and 10 µg /mL) on cell viability (V), Early Apoptosis (EA), Late Apoptosis/Necrosis (LA/N) and Necrosis (N) has been assessed (Figure 27 & 28). A greater concentration of GTEE ([EGCG]= 10 µg /mL) leads to a greater decrease in cell viability ($p = 0.029$), mainly due to an increase in cell death by EA ($p = 0.029$), when compared with the concentration of GTEE ([EGCG]= 5 µg /mL). Regarding, LA/N and N no statistically significant differences were observed between both concentrations.

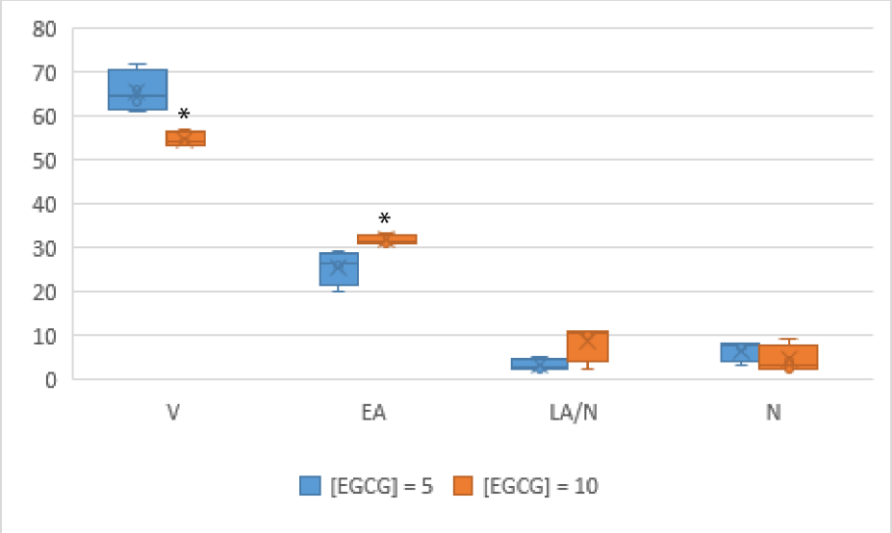


Figure 27. Flow cytometry experiment showing the different kinds of cell death in our experiments (*p< 0.05).

Regardless of the higher cytotoxicity of the 10 µg/mL concentration, the concentration of 5 µg/mL is also highly cytotoxic ($p < 0.05$) and more likely to be reached *in vivo*. For this reason, we opted to use 5 µg/mL to perform the flow cytometry studies.

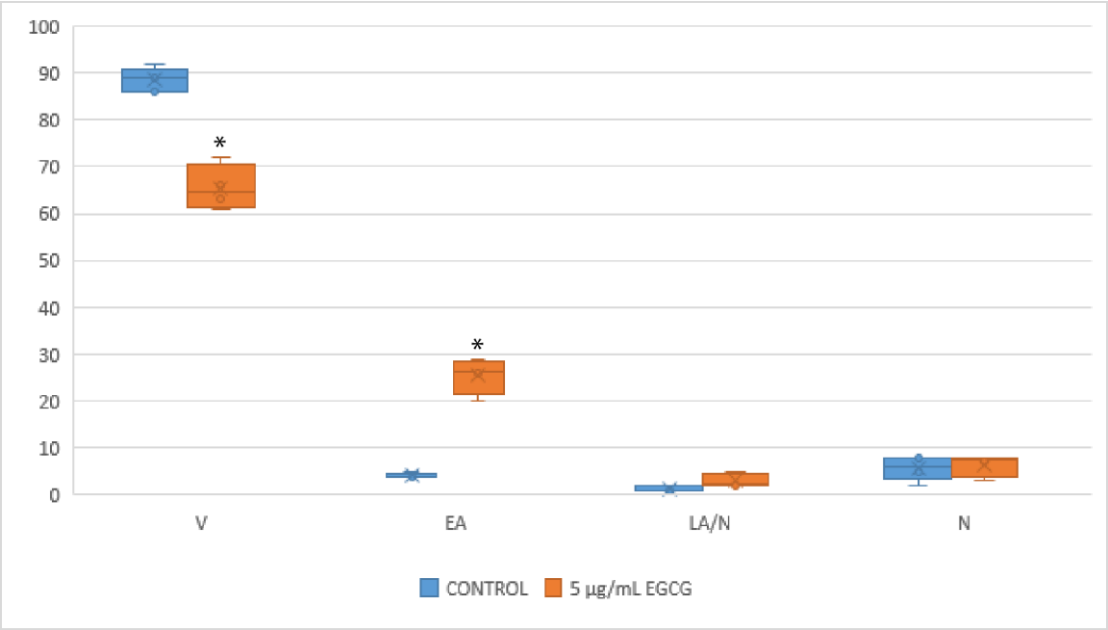


Figure 28. Flow cytometry comparing the cell death type for GTEE ([EGCG]= 5 µg /mL) 5 µg /mL *versus* control (*p< 0.05).

Afterwards, cell death induction by BEVA, FU, IRI, and their combination was studied. Both, when acting alone, and in a therapeutic regimen, as well as when combined with GTEE. The results obtained, as well as statistical analysis of each of the combinations, are summarized in Table 5.

Table 5. Drug *vs* Drug + GTEE.

	GROUP	N	MIN - MAX	MEAN ± SE	MEDIAN [P25 - P75]	P-VALUE
V	Control	5	86 - 92	88,6 ± 1,17	89[86 - 90]	0,016
	GTEE (5)	4	61 - 72	65,5 ± 2,4	64,5[62 - 69]	
	BEVA	4	70 - 77	73,75 ± 1,89	74[70,5 - 77]	0,016
	GTEE + BEVA	5	61 - 68	65,2 ± 1,2	65[65 - 67]	
	FU	4	32 - 40	35,5 ± 1,66	35[33,5 - 37,5]	0,016
	GTEE + FU	5	45 - 53	49 ± 1,52	50[46 - 51]	
	IRI	4	62 - 65	63 ± 0,71	62,5[62 - 64]	0,016
	GTEE + IRI	5	51 - 56	52,4 ± 0,93	52[51 - 52]	
	BEVA + FU + IRI	5	34 - 50	40,8 ± 2,71	41[37 - 42]	0,151
	GTEE + BEVA + FU + IRI	5	43 - 48	45,4 ± 0,81	45[45 - 46]	
EA	Control	5	4 - 5	4,2 ± 0,2	4[4 - 4]	0,016
	GTEE (5)	4	20 - 29	25,5 ± 1,94	26,5[23 - 28]	
	BEVA	4	14 - 23	18,75 ± 2,21	19[15 - 22,5]	0,190
	GTEE + BEVA	5	10 - 20	15,4 ± 1,99	14[13 - 20]	
	FU	4	34 - 45	40,25 ± 2,29	41[37,5 - 43]	0,016
	GTEE + FU	5	12 - 16	13,8 ± 0,73	13[13 - 15]	

Table 5. Drug *Vs* Drug + GTEE (Cont.)

	GROUP	N	MIN - MAX	MEAN ± SE	MEDIAN [P25 - P75]	P-VALUE
	IRI	4	13 - 18	15,25 ± 1,11	15[13,5 - 17]	1,000
	GTEE + IRI	5	13 - 17	15,2 ± 0,66	15[15 - 16]	
	BEVA + FU + IRI	5	23 - 32	29,6 ± 1,69	31[30 - 32]	0,008
	GTEE + BEVA + FU + IRI	5	15 - 17	15,6 ± 0,4	15[15 - 16]	
LA/N	Control	5	1 - 2	1,4 ± 0,24	1[1 - 2]	0,063
	GTEE (5)	4	2 - 5	3 ± 0,71	2,5[2 - 4]	
	BEVA	4	2 - 2	2 ± 0	2[2 - 2]	0,016
	GTEE + BEVA	5	3 - 6	4 ± 0,63	3[3 - 5]	
	FU	4	7 - 13	10,25 ± 1,38	10,5[8 - 12,5]	0,413
	GTEE + FU	5	5 - 10	8,4 ± 0,87	9[9 - 9]	
	IRI	4	4 - 6	5 ± 0,41	5[4,5 - 5,5]	0,016
	GTEE + IRI	5	8 - 10	9 ± 0,32	9[9 - 9]	
	BEVA + FU + IRI	5	7 - 13	9,4 ± 1,03	9[8 - 10]	0,421
	GTEE + BEVA + FU + IRI	5	9 - 10	9,8 ± 0,2	10[10 - 10]	
N	Control	5	2 - 8	5,8 ± 1,11	6[5 - 8]	0,730
	GTEE (5)	4	3 - 8	6,5 ± 1,19	7,5[5 - 8]	

Table 5. Drug *Vs* Drug + GTEE (Cont.)

	GROUP	N	MIN - MAX	MEAN ± SE	MEDIAN [P25 - P75]	P-VALUE
	BEVA	4	5 - 7	5,5 ± 0,5	5[5 - 6]	0,016
	GTEE + BEVA	5	10 - 19	15,4 ± 1,6	16[14 - 18]	
	FU	4	11 - 16	13,75 ± 1,03	14[12,5 - 15]	0,016
	GTEE + FU	5	25 - 33	28,8 ± 1,62	30[25 - 31]	
	IRI	4	15 - 18	16,75 ± 0,63	17[16 - 17,5]	0,016
	GTEE + IRI	5	19 - 24	22,8 ± 0,97	24[23 - 24]	
	BEVA + FU + IRI	5	17 - 24	20,2 ± 1,24	19[19 - 22]	0,008
	GTEE + BEVA + FU + IRI	5	25 - 30	28,2 ± 0,86	29[28 - 29]	

From this set of results, it was possible to observe that, when added to BEVA, GTEE (Figure 29) leads to a decrease in cell viability ($p = 0.016$), through an increase of cell death by LA/N ($p = 0.016$) and N ($p = 0.016$).

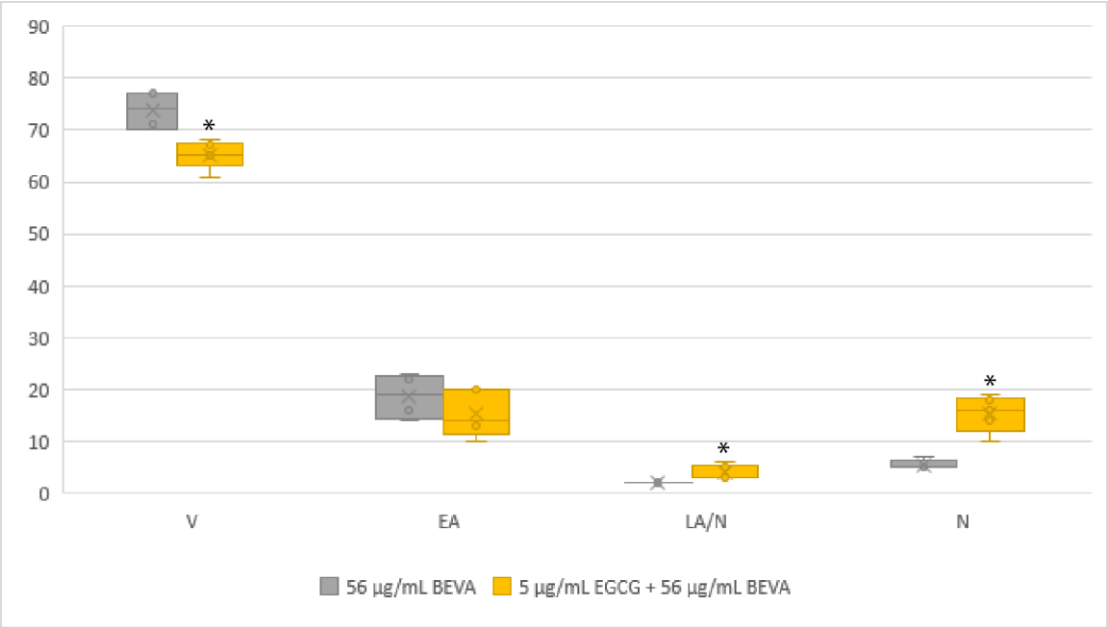


Figure 29. Flow cytometry results comparing BEVA (56 µg /well) and BEVA+ GTEE (56 ng /well + [EGCG]= 5 µg /mL) (*p< 0.05).

Whereas the effect obtained with BEVA + GTEE is promising, the combination of GTEE and FU seems to be severely prejudicial. In fact, treatment with FU (Figure 30) a significant increase of cell viability is seen ($p = 0.016$).

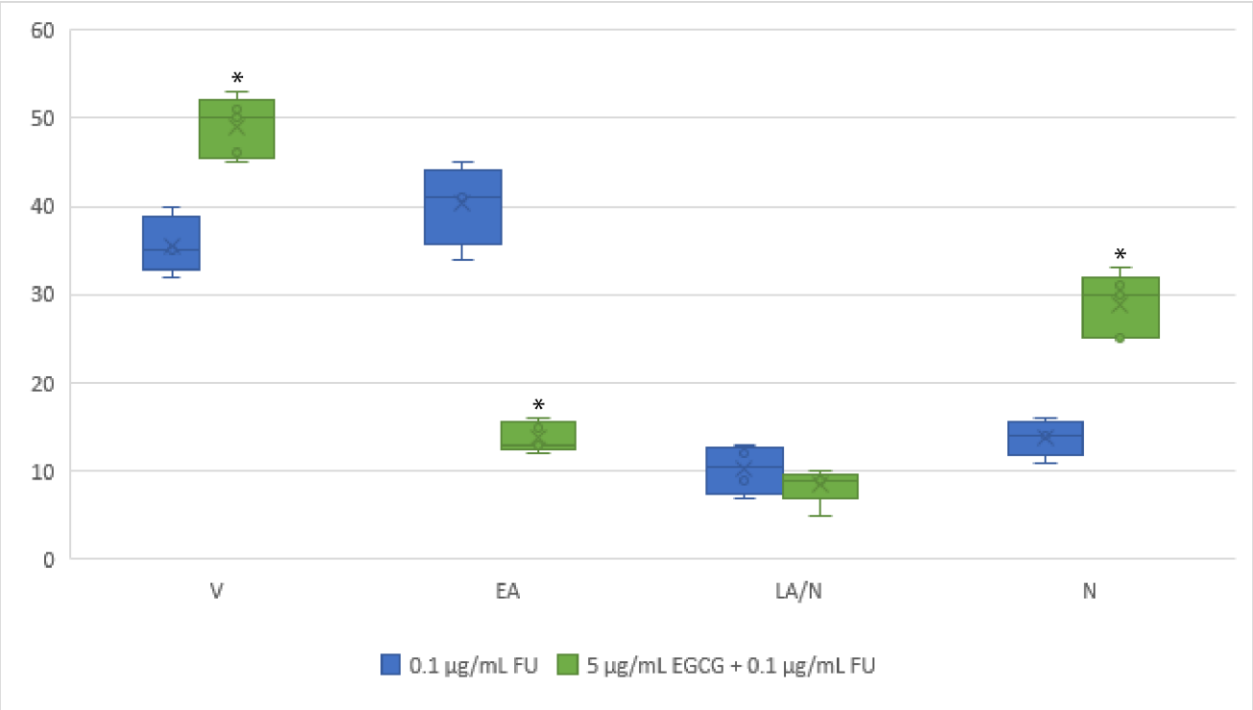


Figure 30. Flow cytometry results comparing FU (0.1 µg /mL) and FU + GTEE (0.1 µg /mL + [EGCG]= 5 µg /mL) (*p< 0.05).

Apparently, the presence of both substances in culture medium makes the rate of EA decrease ($p = 0.016$), compared to cells that exposed only to FU. This decrease in cell death goes along with an increase in death by necrosis ($p < 0.016$).

Regarding the effect of the addition of GTEE to IRI (Figure 31) a decrease in cell viability ($p = 0.016$) was recorded. EA rate remained unchanged and the increase in death was due to increased LA/N ($p = 0.016$) and N ($p = 0.016$).

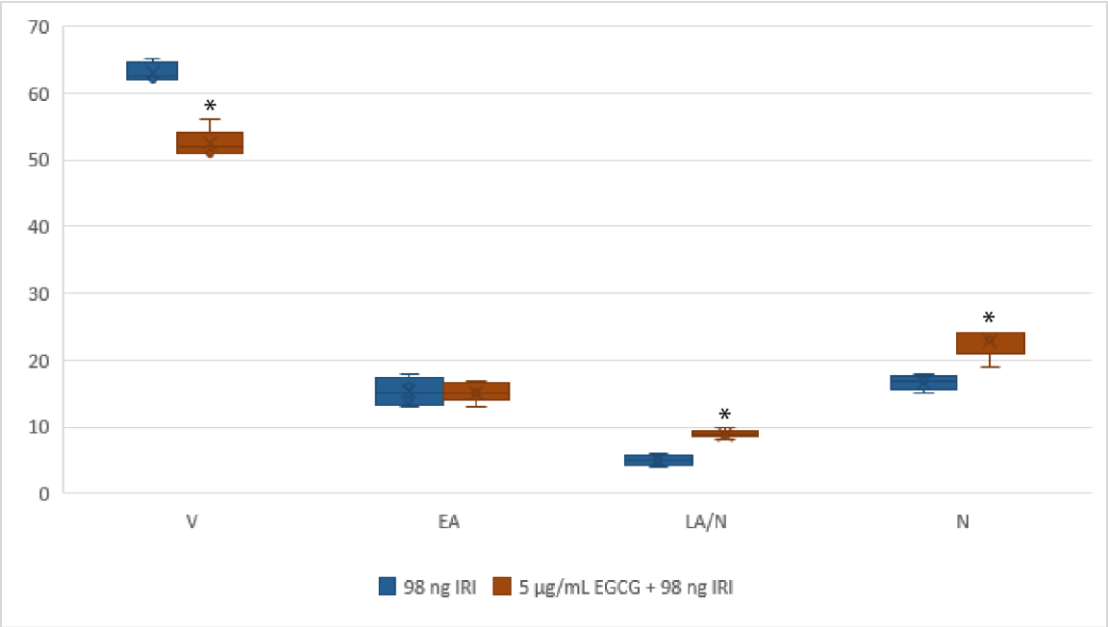


Figure 31. Flow cytometry results comparing IRI (98 ng /well) and IRI+GTEE (98 ng /well + [EGCG]= 5 µg /mL) (*p< 0.05).

Lastly, the effect of the addition of GTEE to a therapeutic regimen consisting of the 3 drugs was studied (Figure 32). This scenario did not produce a change in cell viability ($p > 0.05$). However, a shift in the type of cell death was observed. The combination with GTEE lead to a decrease of EA ($p = 0.008$) and promoted an increase in death by necrosis ($p = 0.008$).

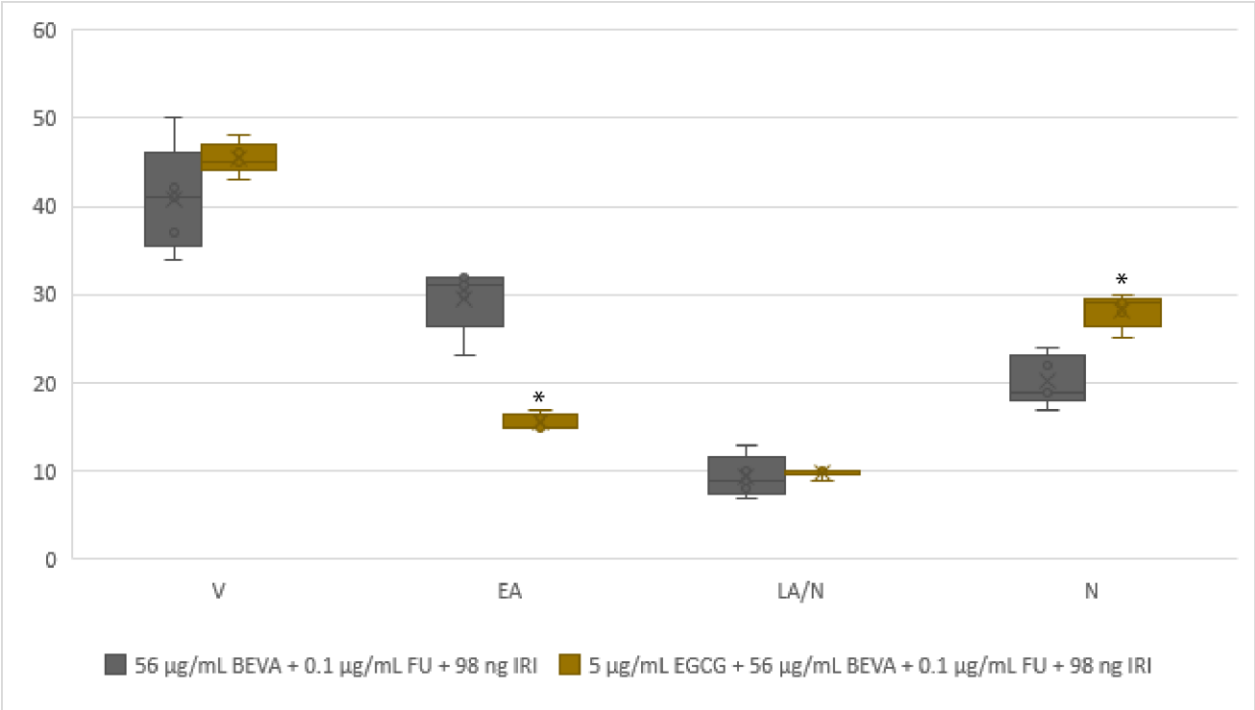


Figure 32. Flow cytometry results comparing BEVA-FIRI (56 ng/well + 0.1 µg /mL + 98 ng/well) and BEVA-FIRI+GTEE (56 ng/well + 0.1 µg /mL + 98 ng/well + [EGCG]= 5 µg /mL) (*p< 0.05).

Eventual cell cycle changes were also analysed by flow cytometry, in order to better understand how the regulation of cell death is being affected not only by GTEE, and the drugs, as well as their combination with GTEE. For this we used a dedicated Propidium Iodide kit (as previously referred).

Firstly, we assessed whether the monotherapy with each of the aforementioned drugs induced cell death at any cell cycle phase.

The results indicated that BEVA does not seem to induce cell arrest at a specific check-point (Table 6).

Table 6. Comparing Control *vs* BEVA.

Sub-G1	G0/G1	S	G2/M
0.548	0.421	0.421	0.151

On the other hand, FU, and IRI monotherapy (Tables 7 & Table 8) induces, preferably, cell proliferation arrest at the S phase level of regulation.

Table 7. Comparing Control *vs* FU (*p< 0.05).

Sub-G1	G0/G1	S*	G2/M
0.421	0.690	0.016	0.690

Table 8. Comparing Control *vs* IRI (*p< 0.05).

Sub-G1	G0/G1	S*	G2/M
1.000	0.151	0.032	0.151

When the effect of GTEE monotherapy on cell cycle regulation was studied, cell arrest took place in all phases of the cell cycle with no statistical different between them (G1, p = 0.008, G0/G1, p = 0.008, S, p = 0.008, and G2-M, p = 0.008).

Afterwards, the effect of GTEE upon cell cycle regulation, of adding GTEE to therapeutic regimens along with the studied substances in terms of cell cycle.

According to the results, when GTEE is added to BEVA monotherapy, cell death takes place mainly in the S phase of the cell cycle (Table 9).

Table 9. Comparing BEVA *vs* GTEE + BEVA (*p< 0.05).

Sub-G1	G0/G1*	S	G2/M
0.151	0.016	0.151	0.222

In regard to the treatment with FU, and with the 3 drugs simultaneously, the addition of GTEE to these regimens induces G0/G1 phase changes (Tables 10 & 11) (GTEE + FU: p = 0.008 and GTEE + BEVA + FU + IRI: p = 0.0016), while maintain the regulation at S phase of the cycle,

previously observed with the FU-monotherapy (GTEE + FU: p = 0.008 and GTEE + BEVA + FU + IRI: p = 0.0016).

Table 10. Comparing FU *vs* GTEE + FU (*p< 0.05).

Sub-G1	G0/G1*	S*	G2/M
0.690	0.008	0.008	0.151

Table 11. Comparing BEVA + FU + IRI *vs* GTEE + BEVA + FU + IRI (*p< 0.05).

Sub-G1	G0/G1*	S*	G2/M
0.063	0.016	0.016	0.413

On the other hand, when GTEE is added to the treatment with IRI, the previously described specificity is lost, and no statistically significant difference is seen between cell death induction in regard to cell cycle phase (Table 12) (p > 0.05).

Table 12. Comparing IRI *vs* GTEE + IRI.

Sub-G1	G0/G1	S	G2/M
0.151	0.151	0.151	0.151

3.3. *In Vivo* studies

An experiment was performed to assess whether GTEE's consumption exerts a protective factor against the proliferation of CRC and its potential as a therapeutic agent.

The selection of the strain 316 (Homozygous) and age-matched wild-type (WT) mice littermates was based on their ideal use for xenograft tumour research.

Figure 33 shows the tumour size variation in rats, previously inoculated subcutaneously with WiDr tumour cells, receiving different types of treatment. The group Cancer received no treatment and the natural tumour growth can be observed. For the remaining groups, time = 0 indicates the day treatment was initiated post-tumour inoculation. The average growth of the tumour in the group receiving no treatment (Cancer) was 5.9 mm^2/d (p < 0.001).

All treatment regimens were able to annihilate the rat tumour (external visual assessment of tumour size = 0 mm^2). The most effective in reducing tumour size was the group Late therapy [-13.46 mm^2/d ($p < 0.001$)], followed by Protection + Therapy [-5,95 mm^2/d ($p = 0.024$)] and Therapy [-2.96 mm^2/d ($p = 0.013$)].

Figure 34 shows the weight variation in rats undergoing different conditions over the course of time (in days). The data was standardised to account for natural weight variation in the control group [0.25 g/d ($p = 0.029$)]. Weight gain was seen in the groups: Cancer [0.15 g/d ($p = 0.516$)]; GTEE [0.53 g/d ($p = 0.005$]; Protection + Therapy [1.13 g/d ($p = 0.004$)]. Weight loss was registered in the groups: Therapy [-0.09 g/d ($p = 0.625$)]; Late therapy [-0.76 g/d ($p < 0.001$)].

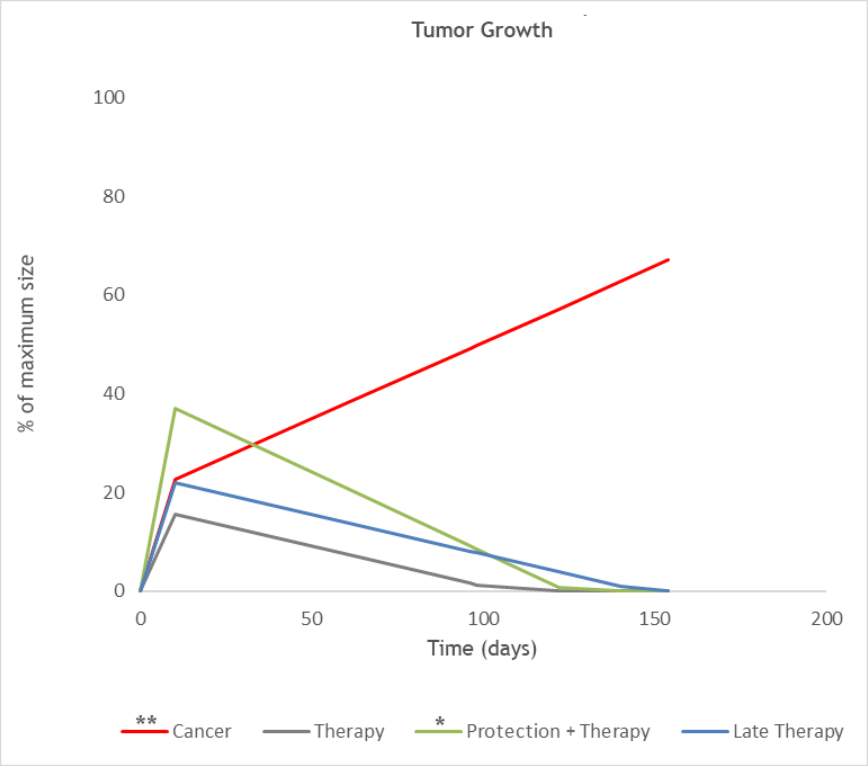


Figure 33. Tumour size variation (mm^2) in rats subjected to different conditions. Cancer - rats inoculated with WiDr cells, received no treatment and drank water; Therapy - rats had only access to GTEE solution after being inoculated with WiDr cells; Protection + Therapy - rats drank solely GTEE-containing solution 2 months prior to tumour inoculation, and continued to drink GTEE solution for the remain of the experiment; Late Therapy - rats were allowed to drink only water for one month after tumour inoculation, after which, water was replaced by GTEE solution.

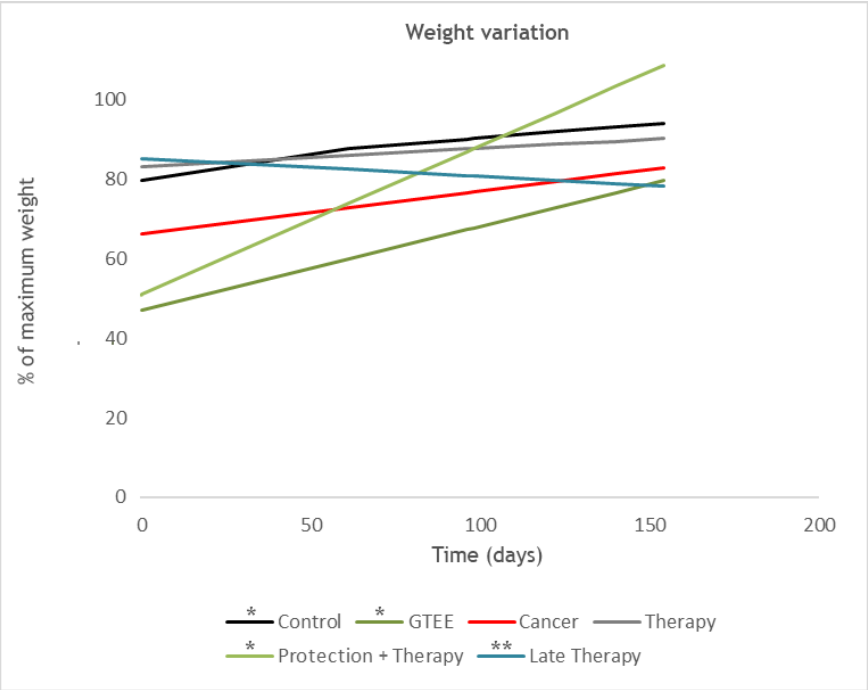


Figure 34. Weight variation (grams) in rats subjected to different conditions Control - rats drank exclusively water; GTEE - rats drank exclusively GTEE-containing solution; Cancer - rats inoculated with WiDr cells, received no treatment and drank water; Therapy - rats had only access to GTEE solution after being inoculated with WiDr cells; Protection + Therapy - rats drank solely GTEE-containing solution 2 months prior to tumour inoculation, and continued to drink GTEE solution for the remain of the experiment; Late Therapy - rats were allowed to drink only water for one month after tumour inoculation, after which, water was replaced by GTEE solution.

3.3.1. Histological study of the organ samples

At the end of each studies animals were sacrificed (using the previously referred protocol) and organs were systematically collected to be processed for routine histology for light microscopy observation (lung, liver, kidney, stomach, small intestine, large intestine, anal area, and tumour/fatty-fibrotic remains when present), according to a well established dissection protocol [31].

Figure 35 shows microsections of an example of: a control normal RNU drinking water (**a**), a control nude RNU drinking water (**b**), a control normal RNU drinking GTEE (**c**), a control nude RNU drinking GTEE (**d**) colored by hematoxylin and eosin stain (H&E).

In all cases, the stomach shows a normal and similar structure, irrespective of the beverage available throughout the study.

Observing images of the stomach of all animals, either controls or animals with tumour (all four groups), it has been concluded that they were all similar. Structure was always normal. An image of the stomach of an animal of GIV (therapy) is shown as an example (Figure 36).

For all the organs collected and processed of all animals, either controls or animals with tumour (all four groups), the structure was normal and similar. As example, it is shown a pair of microsections (control & animal with tumour, regardless of the experimental group) side by side for the other collected areas of the digestive tract: large intestine (Figure 37), small intestine (Figure 38) , anal canal (Figure 39), as well as the other collected organs: lung (Figures 40), liver (Figure 41) and kidney (Figure 42).

An example of one animal per experimental group is shown. Each Figure has two different sections: the photographs illustrate the location/tumour features in each case; the microsections show histologic details (different magnifications with H&E) of the developed/collected tumours (cancer group) or remaining structures at the developing tumour site (therapy, late therapy and protection + therapy groups) (Figure 43-46).

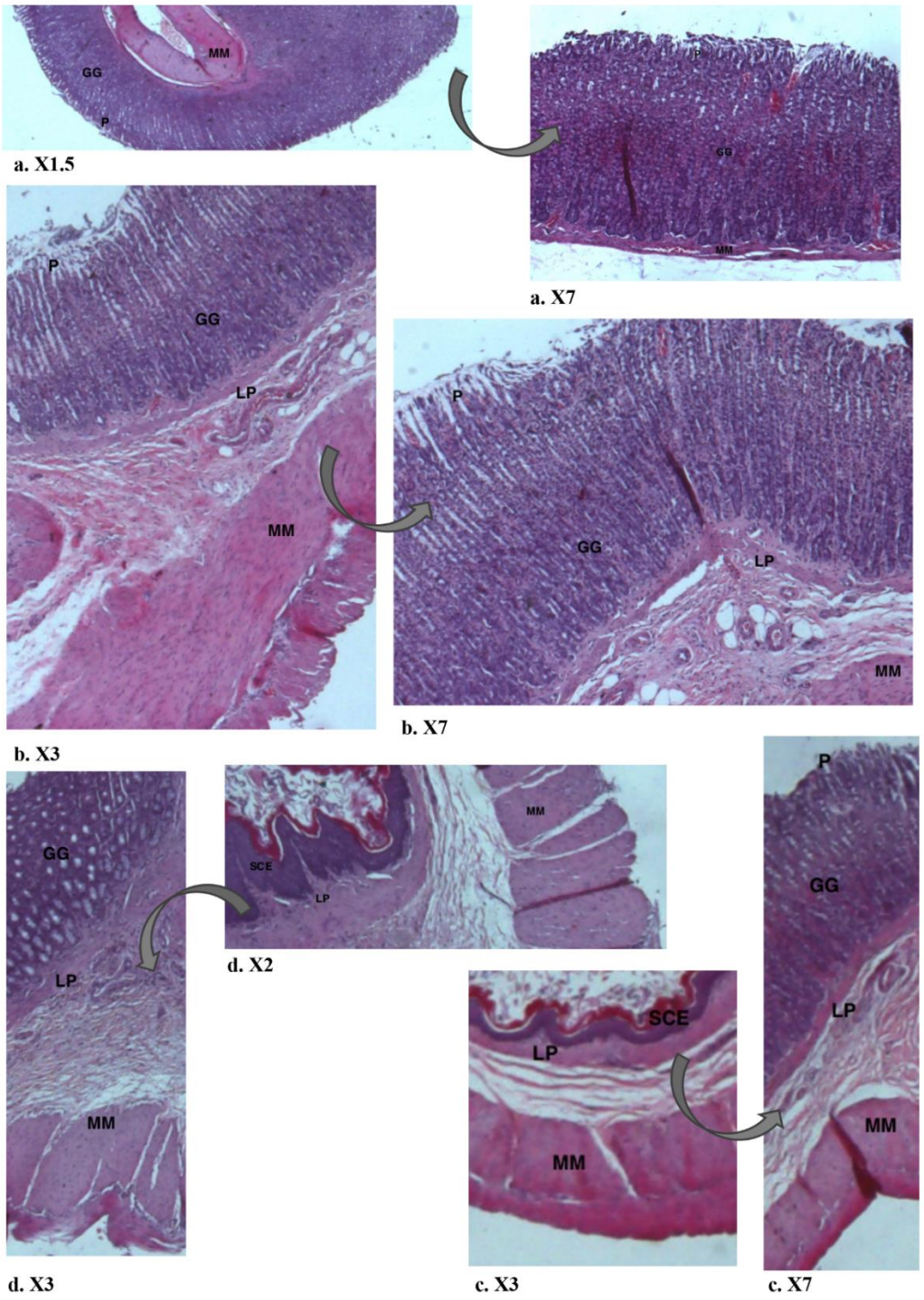


Figure 35. The stomach is a muscular dilation of the digestive tract where mechanical and chemical digestion occurs. A histologic section of the gastric mucosa, which is a simple columnar epithelium (**SCE**), shows the gastric pits (**P**) and glands (**GG**) surrounded by cells of the *lamina propria* (**LP**). The underlying *muscularis mucosae* (**MM**) is also seen. **a.** control normal RNU drinking water; **b.** control nude RNU drinking water; **c.** control normal RNU drinking GTEE; **d.** control nude RNU drinking GTEE, (H&E).

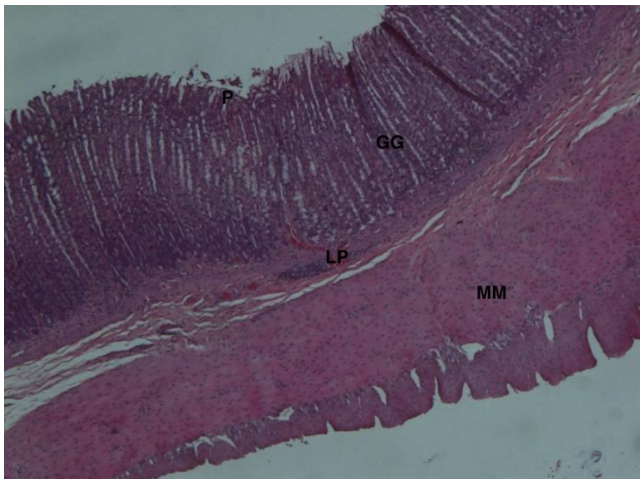


Figure 36. A histologic section of the gastric mucosa of a nude RNU (GIV – therapy), ×5 (H&E).

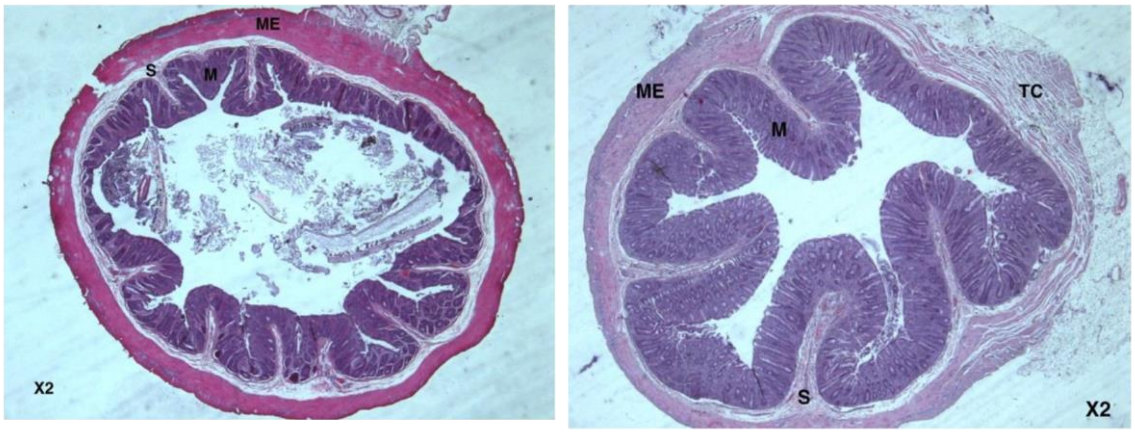


Figure 37. Transverse section of the colon of a normal (**a**) and a nude (**b**) RNU showing the muscularis externa (**ME**), including a tenia coli (**TC**) cut transversely, the submucosa (**S**), and the mucosa (**M**) are filled with tubular intestinal glands (H&E).

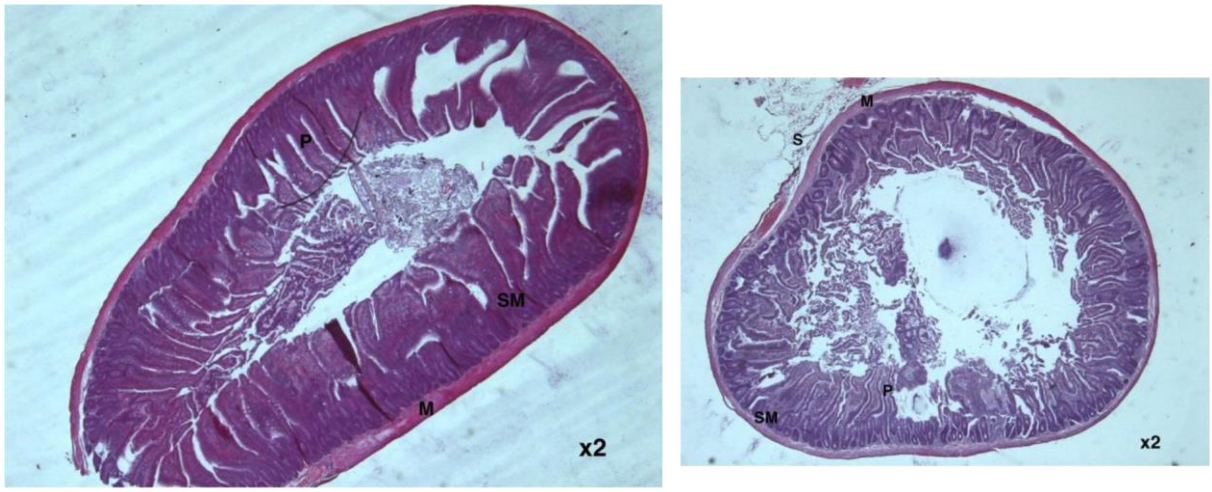


Figure 38. The mucosa and submucosa (**SM**) of the small intestine form distinct projecting folds called plicae (**P**). In this section, the muscularis (**M**) can also be seen as well as part of the outer layer that runs lengthwise: the serosa (**S**), the gut's outer layer: **a.** control, **b.** rat from GIII (protection + therapy) (H&E).

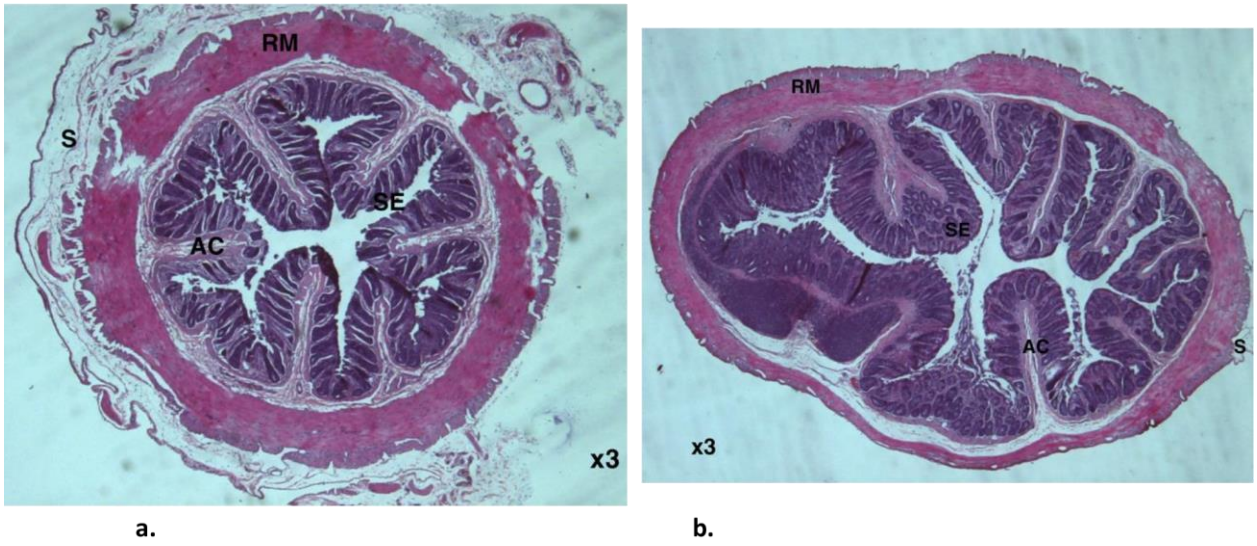


Figure 39. The distal end of the GI tract is the anal canal. The lining of the rectum is a stratified squamous epithelium (**SE**). The mucosa and submucosa of the anal canal form several longitudinal folds, the anal columns (**AC**). The *rectum's muscularis* (**RM**) forms the internal anal sphincter; serosa (**S**) is also seen: **a.** control, **b.** rat from GIII (protection + therapy) (H&E).

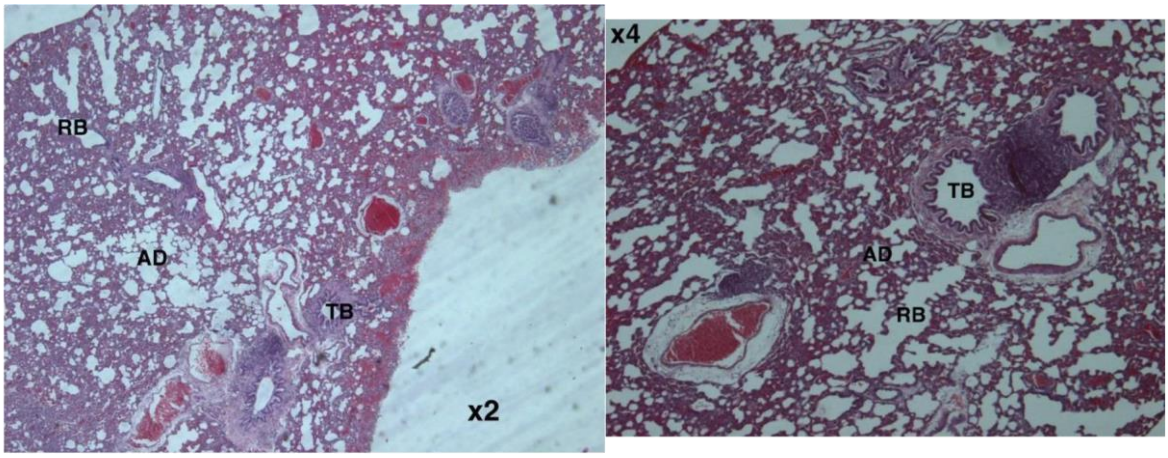


Figure 40. The figures show the branching nature of the air passages: terminal bronchiole (**TB**), respiratory bronchiole (**RB**), alveolar ducts (**AD**): **a.** control, **b.** rat from GIII (protection + therapy) (H&E).

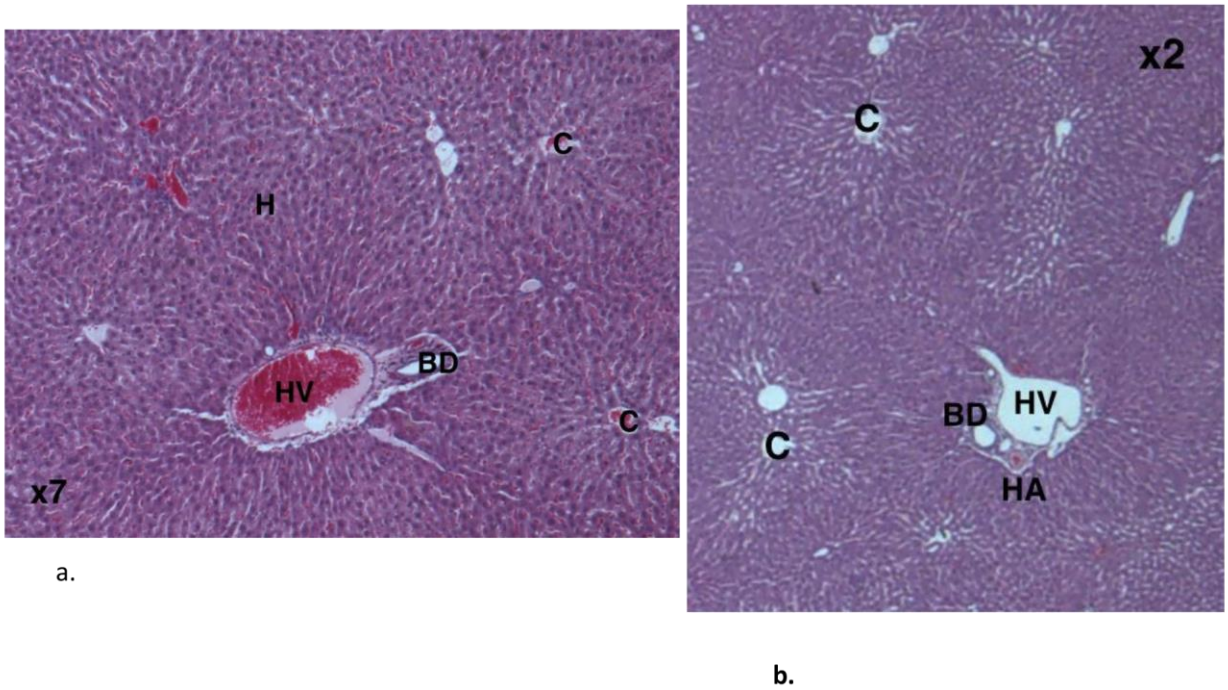


Figure 41. The liver is composed of thousands of polygonal structures called hepatic lobules, which are the basic functional units of the organ. Central veins (***C***), plates of hepatocytes (H), hepatic venule (**PV**), hepatic arteriole (**HA**), and bile ductule (BD) can be seen: a. control, b. rat from GIV (therapy) (H&E).

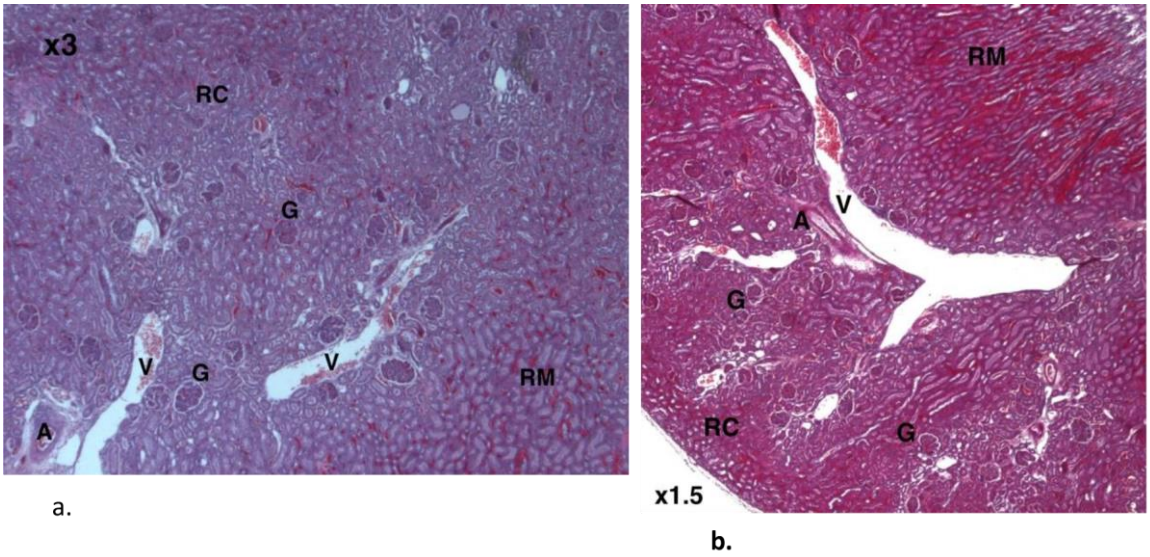
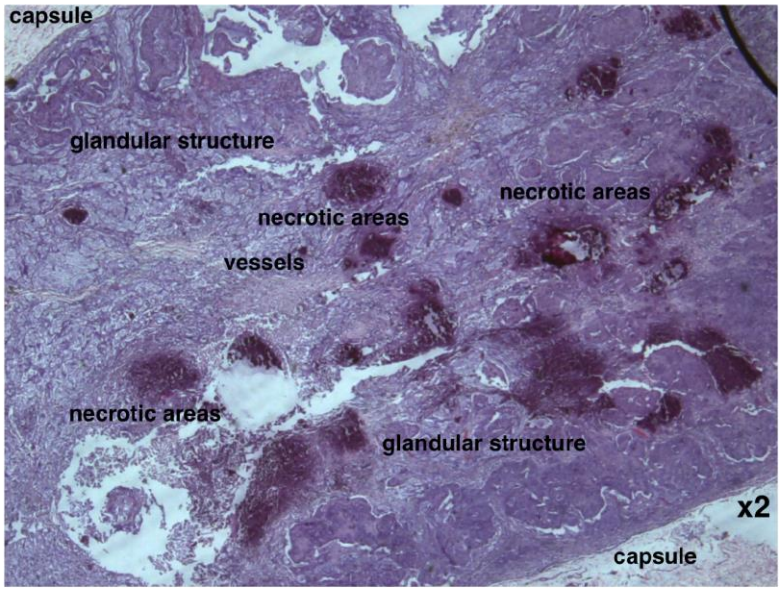
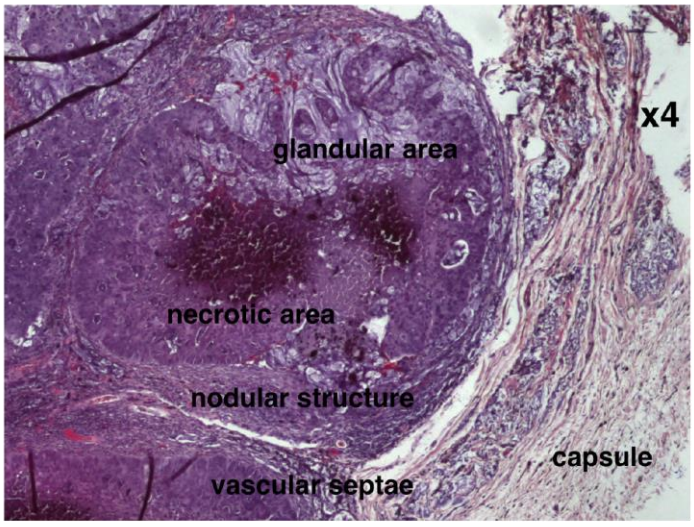


Figure 42. In these sections of the kidney it can be observed the renal cortex (RC) with glomeruli (G), venule (V), and arteriole (A), as well as the renal medullae (RM): **a.** control, **b.** rat from GVI (cancer) (H&E).

A.



B1.



B2.

Figure 43. A. Image pre-collection of the developed tumour (example of GII – Cancer). **B.** Histologic microsections of the collected tumour from the same nude animal: **B1**. it can be seen the vascularized glandular nature of the capsulated tumour showing necrotic areas, x2, **B2.**magnification of a nodular septate area showing its glandular feature with necrotic area, x4 (H&E).

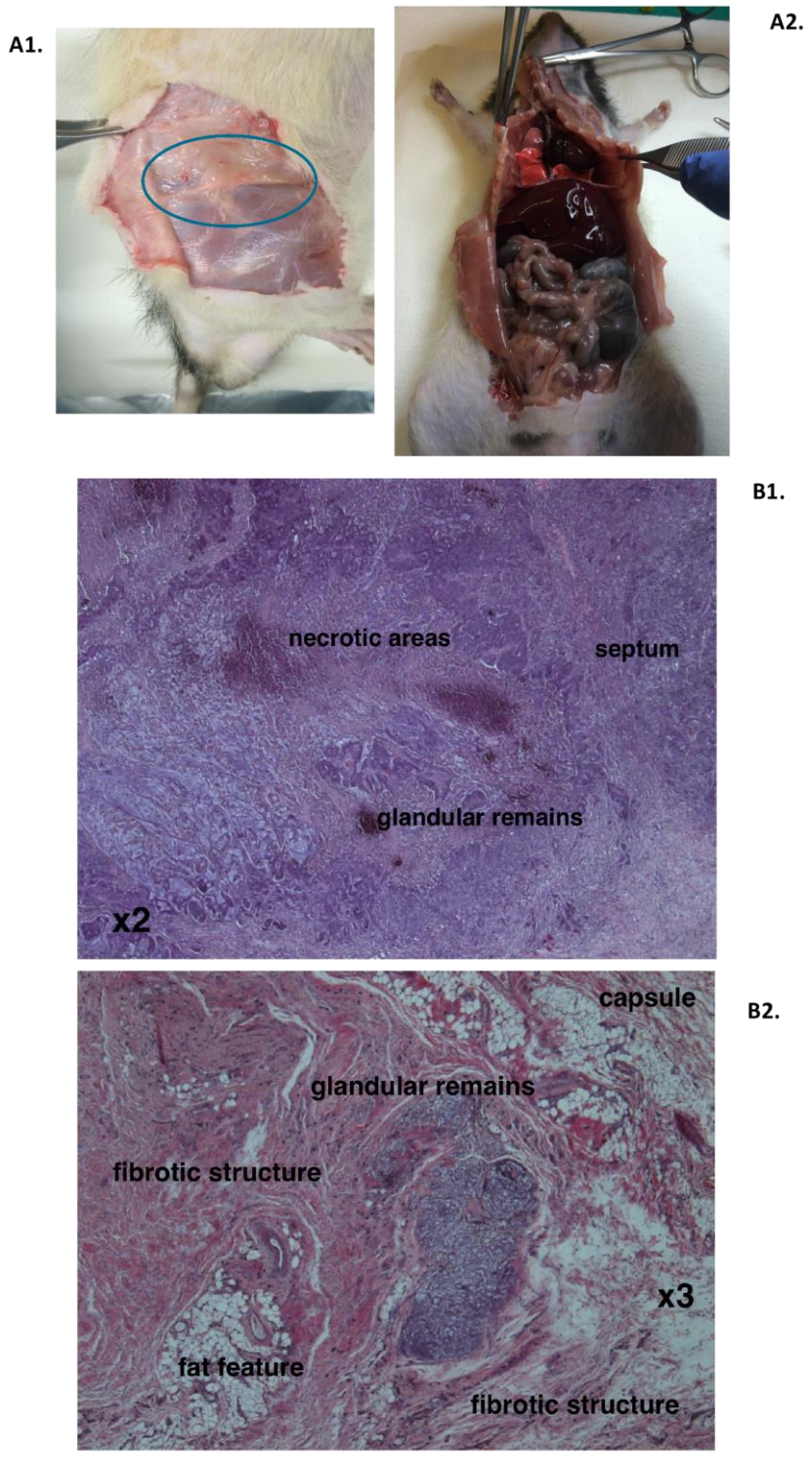
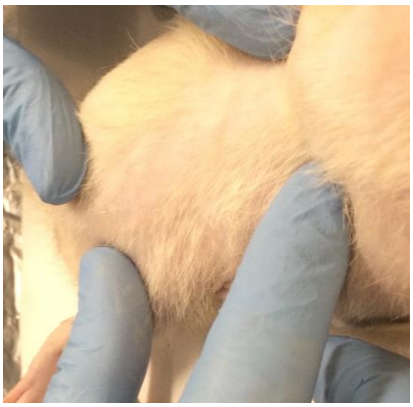
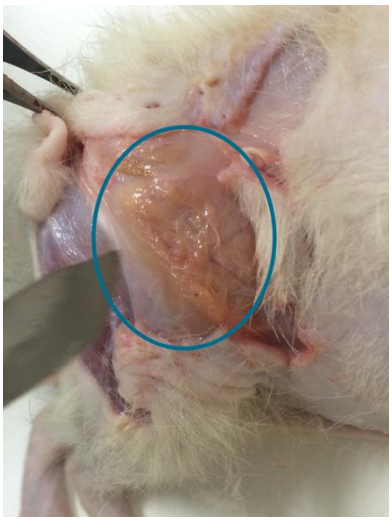


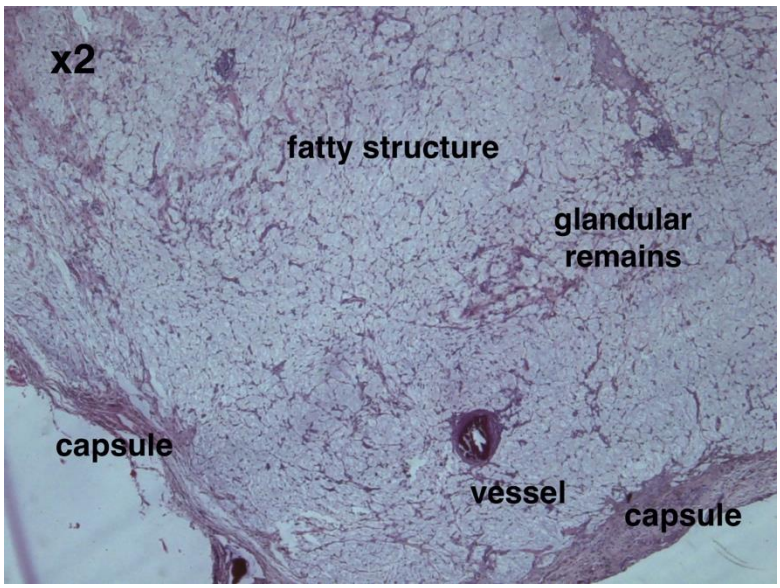
Figure 44. A1. Image at the time of collection of the underskin area where the tumour has developed, not being visible on the exterior (example of GVI – Therapy), **A2**. image of the thoracic and abdominal open areas during the necropsy; **B**. Histologic microsections of the collected tumour from the same nude animal: **B1**. it can be seen the glandular septate nature of the capsulated tumour showing less necrotic areas than GII, x2, **B2.** magnification of a glandular remaining area in between the fibrous and fatty structure, x3 (H&E).

A1.





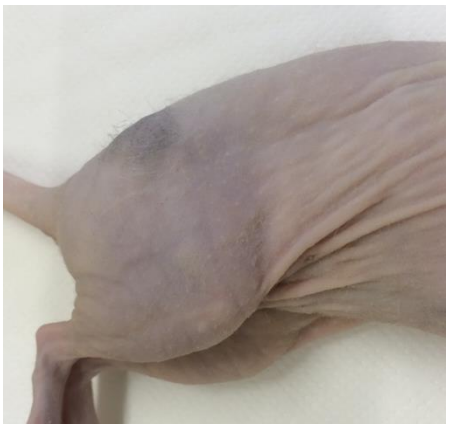
A2.

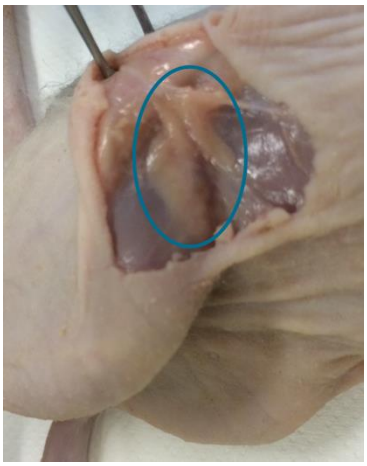


B.

Figure 45. A1. Image pre-collection of the developed tumour – tumour cannot be detected on the exterior (example of GV – late therapy), **A2.** after skin dissection the fatty remainings of the tumour can be observed; **B.** Histologic microsection of the collected tumour from the same nude animal: the vascularized glandular remaining nature of the capsulated tumour embedded in a fatty structure, x2 (H&E).

A1.





A2.

B.

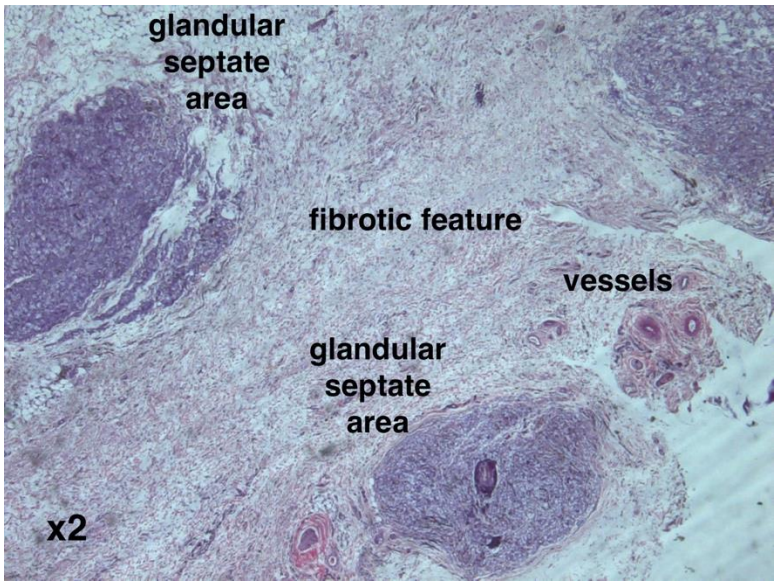


Figure 46. A1. Image at the time of collection of the underskin area where the tumour has developed, not being visible on the exterior (example of GIII – Protection + Therapy), **A2.** after skin dissection the fatty remainings of the tumour can be observed; **B.** Histologic microsection of the collected tumour from the same nude animal: the vascularized glandular remaining nature of the tumour embedded in a fibrotic fatty structure, x2 (H&E).

4. Discussion & Conclusions

Whole plants or mixtures of plants are used in traditional medicine rather than isolated compounds. Evidence has been gathered showing that plant extracts have often higher activity *in vitro* or/and *in vivo* than isolated constituents at an equivalent dose. In the present study, a similar conclusion can be reached, which is shown by the curves of the *in vitro* study using GTEE and EGCG-Pure, respectively. These results are well documented by the *in vivo* experiment [32,33].

Cellular viability of CCD-841 CoN shows no statistically significant difference between groups (Kruskal-Wallis test: $p = 0.095$). In contrast, WiDr cell line exhibits statistically significant cell viability decrease.

Addition of GTEE ([EGCG] = 0.5 μg/Ml) to CET effectively enhances therapy 3.91 times when compared with CET therapy, whilst addition of GTEE ([EGCG] = 5 μg/mL) increases the efficacy 3.35 times. Particularly in this case, GTEE addition in a lower concentration induces a higher death rate. GTEE can enhance the cytotoxicity of therapy with CAP by 2.15, comparing to conventional monotherapy. Addition of GTEE ([EGCG] = 0.5 μg/mL) makes the therapy 2.65 times more effective when compared to monotherapy based upon OX, whilst adding GTEE ([EGCG] = 5 μg/mL) increases treatment efficacy approximately 2.12 times. OX + GTEE ([EGCG] = 0.5 μg/mL) therapy induces a higher rate of cell death compared to OX + GTEE ([EGCG] = 5 μg/mL) therapy. This means that, in this specific case, a lower GTEE concentration is more effective. Addition of GTEE ([EGCG] = 0.5 μg/mL) makes FOLFOX therapy more efficient by approximately 2.66 times, when compared to standard FOLFOX therapy, while adding GTEE ([EGCG] = 5 μg/mL) enhances treatment efficacy by approximately 2.34 times. Furthermore, FOLFOX + GTEE ([EGCG] = 0.5 μg/mL) therapy induces a higher cell death rate, than FOLFOX + GTEE ([EGCG] = 5 μg/mL), thus, in this scenario a lower GTEE concentration is more efficient. GTEE ([EGCG] = 0,5 μg/mL) makes

therapy with CAPIRI approximately 1.90 times more effective when compared with CAPIRI (nowadays used in the clinic), whilst addition of GTEE ([EGCG] = 5 μg/mL) enhances this efficacy 2.27 times. It can be assumed that, in this particular situation, addition of GTEE with a higher concentration induces a higher death rate. Concerning CAPOX, CAPOX + GTEE ([EGCG] = 5 μg/mL) is approximately 1.86 times more effective than sole regimen administration. Combining CET-FOLFIRI with GTEE ([EGCG] = 0.5 μg/mL) induces a significant decrease of cell viability making this therapy 1.24 times higher than CET-FOLFIRI. Adding GTEE ([EGCG] = 5 μg/mL) to CET-FOLFIRI does not induce a significant cell viability decrease. In this case the addition of a lower GTEE concentration might potentiate the efficiency of the combined drug therapy.

BEVA-therapy efficacy does not appear to be altered by GTEE administration. On the other hand, monotherapy with IRI or FU is more effective than IRI + GTEE, or FU + GTEE, in the tested concentrations. Thus, one might say that GTEE addition to IRI or FU decreases the drug's cytotoxic effect in tumoural WiDr cells. Addition of GTEE, in the used concentrations, to FOL does not induce a relevant cytotoxic effect in tumoural cells, being the FOL monotherapy more efficient.

It has been observed by flow cytometry that the concentration of 5 μg/mL is highly cytotoxic for WiDr cells.

Cell death induction by BEVA, FU, IRI, and their combination was studied, both, when acting alone, and in a therapeutic regimen, as well as when combined with GTEE. Added to BEVA, GTEE leads to a decrease in cell viability, through an increase of cell death by LA/N.

Whereas the effect obtained with BEVA + GTEE is promising, the combination of GTEE and FU seems to be severely prejudicial. EA decreases and cell death goes along with an increase in death by necrosis, which is not a good characteristic. Adding GTEE to IRI decreased cell

viability; EA rate remained unchanged and the increase in death was due to increased LA/N. Addition of GTEE to FIRI did not produce a change in cell viability, however a shift in the type of cell death was observed. The combination with GTEE led to a decrease of EA and promoted an increase in death by necrosis, which is not a good feature.

Cell cycle changes were also analysed by flow cytometry, trying to understand how its regulation is being affected not only by GTEE, the drugs, and their combination with GTEE. BEVA does not seem to induce cell arrest at a specific check-point. FU, and IRI monotherapy induced, preferably, cell proliferation arrest at the S phase. With GTEE monotherapy cell arrest took place in all phases of the cell cycle non-specifically. When GTEE is added to BEVA, cell death takes place mainly in the S phase. Addition of GTEE to BEVA + FU + IRI simultaneously, induces G0/G1 phase changes, while maintaining the regulation at S phase. For GTEE + IRI, there is no statistically significant difference between cell death induction in regard to cell cycle phase.

It is uncertain how these pharmacodynamic results would translate into the clinical practice. Based exclusively on the *in vitro* studies performed it cannot be said that GTEs would improve CRC treatment. Should further research work prove this right, then a reduction of chemotherapy dosage might be achieved and thus, also its severe side effects. Terminal patients, left without options, are often willing to try any sort of alternative medicine that might work where all conventional therapy has failed. Nonetheless, it might be wise to discourage consumption of GTEs if a patient is being treated with IRI or FOL.

Regarding *in vivo* experiment, all groups of treatment were successful in reducing the tumours to a too small to measure (TSTS) size, thus making them undetectable to both external inspection and palpation. The rats' weight was not significantly affected by the cancer inoculation nor the type of treatment used.

The survival rate of the rats receiving treatment was 100%, and ocision was performed, not due to worsening health condition of the study elements, but because the desired results had been obtained. Nonetheless, it is worth noting that this study does not include a follow-up study nor the possibility of recurrence or metastasis after tumour remission. The type of treatment that showed the most promising results was the Late Therapy. Despite the excellent results obtained by the use of GTEE as primary therapeutic agent, the question is raised as to whether it would be even more efficient when used as an Adjuvant treatment. Orthotopic tumor models are being explored since they would be suited to test anticancer efficacy of new therapeutics. In this kind of *in vivo* model, tumour cells should be implanted into their organ of origin, since the organ-specific microenvironment will induce a similar tumour growth to the one of the original tumour [34]. Nevertheless, this is not an easy task to achieve and there is not yet a solid background experience in this specific case. It might be a new line of research to compare our subcutaneous induced model.

The research work is ongoing and further studies are being done, not only in vitro and in vivo but also with clinical perspectives.

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