Universidade Estadual de Londrina

ELAINE DA SILVA SIQUEIRA

# ATIVIDADE DA *TRANS*-CHALCONA *IN VITRO* EM LINHAGEM CELULAR HuH7.5 DE CARCINOMA HEPATOCELULAR HUMANO

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Dissertação apresentada ao programa de Pós-Graduação em Fisiopatologia Clínica e Laboratorial da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Fisiopatologia Clínica e Laboratorial.

Orientadora: Prof. Dra. Ivete Conchon-Costa

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Londrina, 02 de agosto de 2019.

Dedico este trabalho aos meus pais, que não mediram esforços para me apoiar nessa etapa tão importante em minha vida.

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“Posso todas as coisas naquele que me fortalece"
Filipenses 4:13

SIQUEIRA, Elaine da Silva. **Atividade da *trans*-chalcona *in vitro* em linhagem HuH7.5 de Carcinoma Hepatocelular humano.** 2019. 62 f. Dissertação (Mestrado em Fisiopatologia Clínica e Laboratorial) – Universidade Estadual de Londrina, Londrina, 2019.


## RESUMO

O carcinoma hepatocelular constitui 70-85% dos tumores primários de fígado, é o sexto câncer com maior incidência em todo o mundo e a quarta causa de morte relacionada ao câncer. Contudo, o tratamento quimioterápico sistêmico é um desafio, devido aos baixos índices de resposta dessas terapias. Neste contexto, a busca por novos compostos com potencial antitumoral e que respondam seletivamente às células cancerígenas tem sido o foco do desenvolvimento de muitos estudos. Dentre eles, a *trans*-chalcona (TC) (1,3-diphenyl-2-propen-1-one), um composto precursor dos flavonóides que apresenta algumas funções biológicas, como antioxidantes anti-inflamatórios e inibição da proliferação de células tumorais humanas. Deste modo, o objetivo deste trabalho foi investigar a ação antitumoral da *trans*-chalcona (TC) sobre linhagem HuH7.5 de carcinoma hepatocelular (CHC). As células tumorais HuH7.5 foram tratadas com TC em concentrações crescentes (12,5-100 μM) nos tempos 24 e 48 horas, a viabilidade celular foi verificada por MTT e a concentração inibitória de 50% das células ($IC_{50}$ 23,66 μM) foi determinada em 48 horas. Foram quantificados a proliferação celular por azul de tripan e microscopia óptica, produção de EROs, despolarização mitocondrial e autofagia, além da análise do ciclo celular e apoptose pelo analisador de células Muse® e marcações imunocitoquímicas de p-p53 e β-catenina. Os dados mostraram uma ação TC-citotóxica eficaz de maneira dependente da dose e do tempo em baixas concentrações micromolares sem causar toxicidade para células não-cancerígenas, como eritrócitos. O tratamento com TC causou danos na membrana mitocondrial, afetando a expressão do gene supressor de tumor (p-53) e da via de desenvolvimento do tumor (β-catenina), induzindo a morte celular por autofagia. Além disso, o TC diminuiu a capacidade metastática do HuH7.5, interferindo na capacidade de migração / invasão desse tipo de célula.



**Palavras-chave:** Carcinoma Hepatocelular. Células HuH7.5. *trans*-chalcona. EROs. Despolarização mitocondrial. Autofagia. p-53. β-catenina.

SIQUEIRA, Elaine da Silva. **Activity of trans-chalcone in vitro in human hepatic carcinoma HuH7.5 cell line.** 2019. 62 p. Dissertation (Master's degree in Clinical and Laboratory Physiopathology) - Londrina State University, Londrina, 2019.

## ABSTRACT

Hepatocellular carcinoma accounts for 70-85% of primary liver tumors, the sixth most common cancer in the world, and the fourth leading cause of cancer-related death. However, systemic chemotherapy is a challenge because of the low response rates of these therapies. In this context, the search for new compounds with antitumor potential and that respond selectively to cancer cells has been the focus of many studies. Among them, *trans*-chalcone (TC) (1,3-diphenyl-2-propen-1-one), a flavonoid precursor compound that exhibits some biological functions, as anti-inflammatory antioxidants and inhibition of human tumor cell proliferation. Thus, the objective of this work was to investigate the antiproliferative action of *trans*-chalcone (TC) on cells of the HuH7.5 line of hepatocellular carcinoma (HCC). HuH7.5 tumor cells were treated with TC at increasing concentrations (12.5-100 μM) at 24 and 48 hours, cell viability was verified by MTT and 50% inhibitory concentration of cells ($IC_{50}$ 23.66 μM) was determined within 48 hours. Trypan blue proliferation and light microscopy, ROS production, mitochondrial depolarization and autophagy, cell cycle analysis and apoptosis by the Muse® cell analyzer and immunocytochemical markings of p-p53 and β-catenin were quantified. The data showed an effective dose- and time-dependent TC-cytotoxic action at low micromolar concentrations without causing toxicity to non-cancerous cells such as erythrocytes. TC-treatment caused mitochondrial membrane damage, affecting tumor suppressor gene expression (p-53) and tumor development pathway (β-catenin), inducing cell death by autophagy. In addition, TC decreased the metastatic capacity of HUH7.5, interfering with the migration / invasion capacity of this cell type.

**Key words:** Hepatocellular carcinoma. HuH7.5 cells. *trans*-chalcone. EROs. Mitochondrial depolarization. Autophagy. p-53. β-catenin.

## LISTA DE ILUSTRAÇÕES

**Figura 1** – Fatores etiológicos do Carcinoma Hepatocelular ................................. 15
**Figura 2** – Classificação da Clínica de Câncer Hepático de Barcelona .................. 16
**Figura 3** – Vias moleculares da hepatocarcinogênese ........................................... 18
**Figura 4** – Estrutura química da Chalcona ............................................................ 21
**Figura 5** – Estrutura da *trans*-Chalcona.................................................................. 23

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| 3MCIC | trimetoxifenil e isatinil |
| ADMET | absorção, distribuição, metabolismo, excreção e toxicidade |
| ALC | Alcoolização Tumoral Percutânea |
| ALT | alanina aminotransferase |
| ANT | antraciclinas |
| ARF | ablação por radiofrequência |
| AST | aspartato aminotransferase |
| BCLC | do inglês *Barcelona Clinic Liver Cancer* |
| CH1 | 2,3,4-trimetoxi-2'-hidroxi-chalcona |
| CH2 | 3'-bromo-3,4-dimetoxi-chalcona |
| CHC | Carcinoma Hepatocelular |
| c-kit | proteína c-KIT |
| c-Myc | do inglês *cell myelocytomatosis* |
| COX | ciclooxigenases |
| CSCs | Células-tronco Cancerígenas |
| DDTs | Diretrizes Diagnósticas e Terapêuticas |
| DNA | ácido desoxirribonucleico |
| Doxo | doxorrubicina |
| EROs | espécies reativas de oxigênio |
| FAL | fosfatase alcalina |
| FOLFOX | fluorouracil, ácido folínico e oxaliplatina |
| GemOx | gencitabina e oxaliplatina |
| GLOBOCAN | do inglês *New global cancer data* |
| HBV | vírus da hepatite B |
| HCV | vírus da hepatite C |
| hERG | do inglês *Ether-a-go-go-Related Gene* |
| Hh | Hedgehog |
| IARC | do inglês *International Agency for Research on Cancer* |
| IFN-γ | interferon-γ |
| IGF-2 | fator de crescimento semelhante à insulina 2 |
| IHH | do inglês *immortalized human hepatocyte* |
| I-kB | proteína inibitória de kappa B |

| | |
|---|---|
| IKK | Complexo quinase |
| INCA | Instituto Nacional de Câncer |
| JAK | Janus kinase |
| k-ras | do inglês *Kirsten rat sarcoma viral* oncogene *homolog* |
| NF-κB | factor nuclear kappa B |
| NO | óxido nítrico |
| OMS | Organização Mundial de Saúde |
| OPAS | Organização Pan-Americana da Saúde |
| PDGF | Fator de Crescimento derivado de Plaquetas |
| PGE2 | prostaglandina E2 |
| PIAF | cisplatina, interferon-α e fluorouracil |
| PST | do inglês *Performance Status Test* |
| Raf | oncogene Raf |
| RAF | proteínas quinase específicas de serina/treonina |
| RNA | ácido ribonucleico |
| STAT | do inglês *activator of transcription* |
| TACE | do inglês *transarterial chemoembolization* |
| TC | *trans*-chalcona |
| TGF-α | Fator de Crescimento Transformante alfa |
| TICs | Células Iniciadoras de Tumor do fígado |
| TNF-α | Fator de Necrose Tumoral-α |
| VEGF | Fator de Crescimento do Endotélio Vascular |

**SUMÁRIO**

**1 INTRODUÇÃO** .......... 13
1.1 ASPECTOS GERAIS DO CÂNCER .......... 13
1.2 CARCINOMA HEPATOCELULAR .......... 14
1.2.1 Hepatocarcinogênese .......... 16
1.2.2 Tratamentos .......... 19
1.3 CHALCONAS .......... 21
1.3.1 *trans*-Chalcona .......... 23

**2 REFERÊNCIAS** .......... 26

**3 OBJETIVOS** .......... 33
3.1 OBJETIVO GERAL .......... 33
3.2 OBJETIVOS ESPECÍFICOS .......... 33

**4 PRODUÇÃO CIENTÍFICA** .......... 34
4.1 ARTIGO: *Trans*-chalcone induces death by autophagy mediated by p-53 up-regulation and β-catenin down-regulation on human Hepatocellular Carcinoma HuH7.5 line. .......... 35

# 1. INTRODUÇÃO

## 1.1 ASPECTOS GERAIS DO CÂNCER

O câncer é caracterizado pela desregulação da proliferação celular em diferentes tipos celulares com capacidade de invasão de tecidos e órgãos, os quais induzem a instabilidade genômica (HOLTZ, 2009; LEIPELT, 2016; CONNOR, 2015).

Seu processo de desenvolvimento ocorre por três estágios. A primeira fase denominada iniciação, pode ter início de forma espontânea ou ser impulsionado por mutações e forças seletivas por meio da ação de agentes carcinogênicos biológicos, físicos ou químicos (RIBEIRO, 2014; PIRETTO et al., 2019; MARRASSINI & ANESINI, 2016). No estágio de promoção, a exposição à agentes promotores, como determinadas substâncias presentes no ambiente ou alguns medicamentos como hormônios sexuais irão atuar nas células já alteradas na iniciação e, na última fase, ocorre a progressão descontrolada das células cancerígenas que adquirem mutações genéticas e transformam-se em malignas com consequente metástase (INCA, 2011).

Portanto, a partir dos diversos tipos de estímulos que a célula recebe, o sistema imunológico entra em ação e são expressos uma variedade de fenótipos e desencadeiam inúmeras alterações no ácido desoxirribonucleico (DNA), como substituições das bases nitrogenadas, inserções ou deleções de segmentos pequenos ou grandes de DNA, rearranjos, amplificação gênica, os quais levam às mutações de genes-chave de uma célula normal (STRATTON, 2009; PIRETTO et al., 2019). Essas mutações genéticas atuam nos genes promotores de crescimento (proto-oncogenes), que estão inativos em células normais e após estímulos se transformam em oncogenes, os quais são responsáveis pela malignização das células normais que acabam por invadir tecidos e levar à metástase (RIBEIRO, 2014; INCA, 2019). Além disso, também podem ativar os genes inibidores do crescimento (supressores de tumor) e os genes de reparo de DNA.

Segundo a Organização Mundial de Saúde (OMS), em 2018 o câncer foi responsável por 9,6 milhões de óbitos, sendo considerada a segunda principal causa de morte no mundo (OMS, 2018).

Conforme dados divulgados pela *International Agency for Research on Cancer* (IARC, 2018), a sequência dos dez cânceres com maior incidência no mundo é: pulmão (2,09 milhões de casos), mama (2,08 milhões), colorretal (1,8 milhão),

próstata (1,28 milhões), estômago (1,03 milhões), fígado (841 mil), esôfago (572 mil), colo uterino (569 mil), tireóide (567 mil) e bexiga (549 mil) (IARC, 2018). Já as principais causas de morte estão relacionadas ao câncer de pulmão (1,76 milhão), colorretal (862 mil), estômago (783 mil), fígado (782 mil) e mama (627 mil) (OPAS, 2018).

No Brasil, a estimativa 2018-2019 é de 217,2 novos casos de câncer para cada 100.000 habitantes. Nos homens a maior incidência é para câncer de próstata (74,0/100.000), seguido dos cânceres de pulmão (18,16/100.000) e colorretal (16,83/100.000). Nas mulheres, há maior ocorrência de câncer de mama (56,66/100.000), seguido de câncer colorretal (17,90/100.000) e colo de útero (15,43/100.000) (INCA, 2018).

## 1.2 CARCINOMA HEPATOCELULAR

O hepatocarcinoma ou carcinoma hepatocelular (CHC) constitui 70-85% dos tumores primários de fígado, segundo a *International Agency for Research on Cancer* (IARC), é o sexto câncer com maior incidência em todo o mundo e a quarta causa de morte relacionada ao câncer (OPAS, 2018; GOMES et al., 2013; CAMPOS, 2017), representando um importante problema mundial de saúde pública, pois não tem uma taxa de recuperação bem-sucedida e sua incidência vem aumentando a cada ano. Segundo a OMS, em 2008 foi registrado 694 mil óbitos no mundo e em 2018 a taxa de mortalidade chegou a 781.631 (AMORIM & MERCHÁN-HAMANN, 2013; GLOBOCAN, 2018).

Embora no Brasil a incidência seja baixa, a ocorrência tem sido constante ao longo dos anos. Foi observado entre 1988 e 1991 uma taxa de incidência de 1,07 a 9,43/100.000 nos homens e de 0,28 a 7,04/100.000 em mulheres, dependendo da região do país, em 2018 a média de incidência para homens foi de 6,3 e para mulheres 3,4/100.000, (IARC, 2018). Com relação à mortalidade em nosso país, em 2015 foram registrados 9.711 óbitos (INCA, 2015) e em 2017 esse número chegou à 10.200 (INCA, 2018).

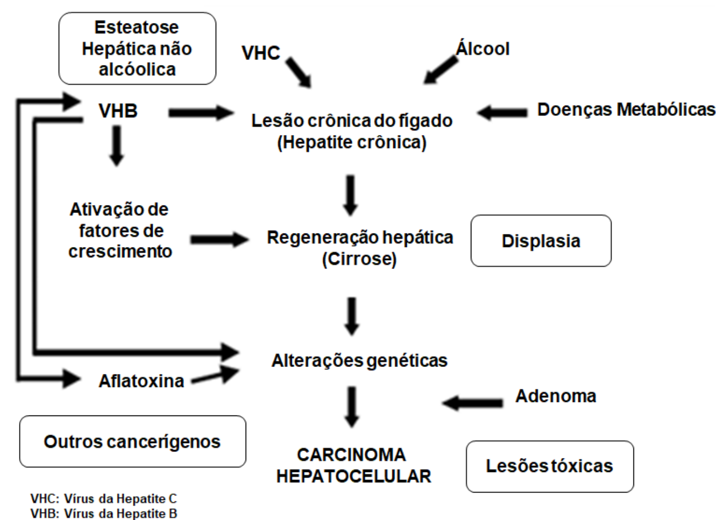
Em pesquisa nacional que incluiu dados de 29 centros para um total de 1.405 pacientes com diagnóstico de CHC, verificou-se que a idade média foi de 59 anos e 78% dos casos eram do sexo masculino, sendo a cirrose hepática presente em 98%

dos casos e a infecção crônica pelo vírus da hepatite C (HCV) a etiologia mais comum (54%), seguido pelo vírus da hepatite B (HBV) (16%) e álcool (14%) (ARIEDE, 2017).

Os tumores malignos de fígado podem ser divididos em dois tipos: carcinoma primário, que tem sua origem no próprio órgão, como o colangiocarcinoma e o carcinoma hepatocelular (CHC); e o carcinoma secundário ou metastático, originado em outro órgão e posteriormente acometendo o fígado (ARIEDE, 2017), sendo o CHC muito agressivo, com elevado índice de mortalidade e com a expectativa de vida média inferior a um mês, após o início dos sintomas como icterícia e ascite (GOMES et al., 2013).

Fatores etiológicos são bem definidos no CHC, como hepatite crônica devido à infecção persistente pelos vírus da Hepatite B (HBV) ou Hepatite C (HCV), consumo excessivo de álcool, complicações do quadro de cirrose hepática dentre outros fatores, de acordo com a **Figura 1**, levando a proliferação desordenada dos hepatócitos (RIBEIRO, 2014; GUERRA, 2014).

**Figura 1:** Fatores etiológicos do CHC

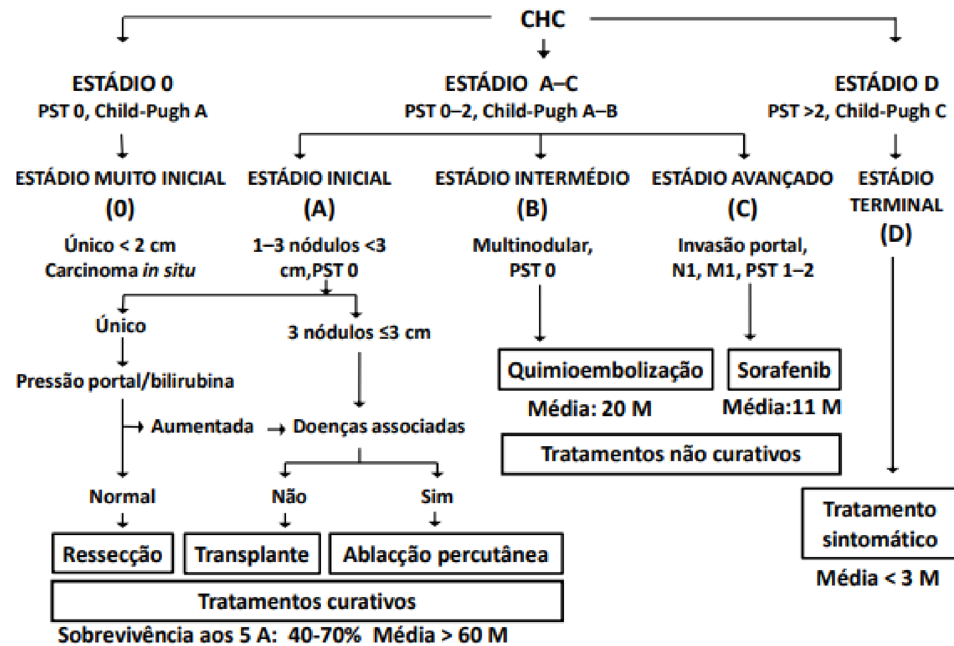



**Fonte:** Adaptado de: Conte (2000)

O estadiamento do CHC é definido de acordo com o tamanho e localização do tumor, os sintomas e a função hepática. Existem diversos sistemas de estadiamento de CHC, atualmente, a metodologia de escolha da maior parte das instituições é o das Diretrizes Diagnósticas e Terapêuticas (DDTs) para CHC, desenvolvida pelo *Barcelona Clinic Liver Cancer group* (BCLC), que divide os pacientes de CHC em 5 estágios (BCLC 0: muito precoce, BCLC A: precoce, BCLC

B: intermediário, BCLC C: avançado e BCLC D: terminal) conforme ilustrado na **Figura 2** (BRUIX e SHERMAN, 2011; SIMÃO, 2014).

**Figura 2:** Classificação da Clínica de Câncer Hepático de Barcelona




**Fonte:** Simão (2014)

### 1.2.1 Hepatocarcinogênese

A hepatocarcinogênese compreende múltiplas e complexas vias de estímulos extrínsecos conforme **Figura 3**, que induzem alterações gênicas nos hepatócitos maduros em células iniciadoras de tumor do fígado (TICs) ou células-tronco cancerígenas (CSCs), seja pela desdiferenciação ou transformação de progenitores do fígado levando a morte celular ou sucessiva proliferação resultando em populações monoclonais (WU et al., 2018; CHARNI et al., 2014; APOLINÁRIO, 2017). As alterações gênicas incluem mutações, translocações, amplificação ou perda de cromossomo resultando na ativação de oncogenes ou diminuição de atividade de genes supressores tumorais (APOLINÁRIO, 2017).

Deste modo, Shiraha et al. (2013) classificaram e subdividiram didaticamente as principais alterações e mutações do CHC em: inativação de supressores tumorais (p53 e Retinoblastoma), ativação de oncogenes (c-Myc, k-ras, Raf), reativação de vias de desenvolvimento (Wnt/β-catenina e Hedgehog (Hh) e, ativação de fatores de

crescimento e seus receptores (IGF-2; TGF-α) (APOLINÁRIO, 2017; GUERRA, 2014; ZUCMAN-ROSSI, 2010).

O gene TP53 é localizado no braço curto do cromossomo 17 e dá origem a proteína p53 (17p13.1), o qual apresenta inúmeras funções devido a sua capacidade de ligar-se a sequências específicas do DNA e atuar como fator de transcrição multifuncional regulando positivamente ou negativamente a expressão de diversos genes envolvidos nas vias de sinalização celulares, como por exemplo, morte celular programada, parada do ciclo celular, diferenciação e senescência celular, atuando dessa forma como "guardiã do genoma" contra o estresse genotóxico (KLUMB & CAVALCANTE JUNIOR, 2002; GUERRA, 2014; CHARNI et al., 2014; HAINAUT & HOLLSTEIN, 1999).

A ativação do gene supressor tumoral p53 pode ser decorrente da diversidade de sinais como em resposta à danos no DNA, estresse oxidativo, hipóxia, privação de nutrientes e ativação oncogênica (APOLINÁRIO, 2017; CHRISTOPHOROU et al., 2005). Já a sua inativação responde por até 60% dos casos de CHC, esta inativação pode se dar por mutações no gene p53, sendo o tipo *missense* o mais frequente, pois ocorre troca de um nucleotídeo, promovendo alteração da função normal da proteína, aumentando sua meia vida e acumulando-se nas células tumorais por sua vez mutação do tipo *nonsense*, embora em menor frequência, ocasiona deleções de porções do gene ou inserções de nucleotídeos que levam a parada da leitura do ácido ribonucleico (RNA) mensageiro originando a proteína não funcional (KLUMB & CAVALCANTE JUNIOR, 2002).A ativação desse gene na célula tumoral funciona como uma molécula *checkpoint* da proliferação em resposta ao dano do DNA e está diretamente relacionada ao bloqueio do ciclo celular da fase G1/S, além de induzir a expressão de alguns genes transcricionais como p21, Bax-1 e MDM-2, impedindo dessa forma, a replicação do DNA e ocasionando morte celular (KLUMB & CAVALCANTE JUNIOR, 2002; GUERRA, 2014; CHARNI et al., 2014).

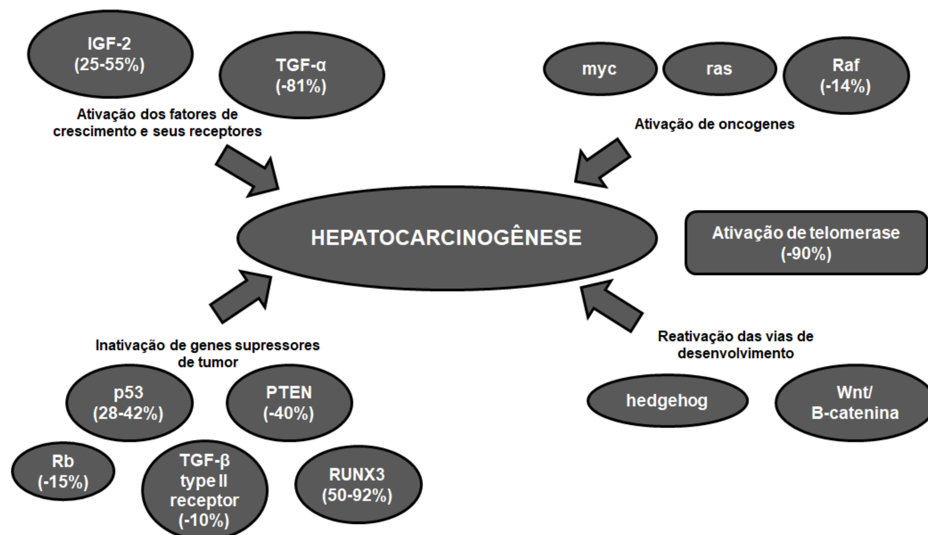
Quanto às vias de desenvolvimento, a proteína β-catenina é uma proteína bifuncional codificada pelo gene CTNNB1 da família das proteínas cateninas que possui funções sinalizadoras e estruturais, atuando na regulação da coordenação de adesão célula-célula e a transcrição genética (VALENTA et al., 2012; BOURROUL et al., 2016). Quando localizada na membrana citoplasmática apresenta papel crucial no balanceamento da adesão celular e, quando livre no citoplasma é responsável pela via de sinalização de Wnt/β-catenina, pois a β-catenina é uma subunidade do

complexo proteico de caderina o qual atua como transdutora do sinal intracelular na via de sinalização Wnt (GUERRA, 2014; VALENTA et al., 2012; BOURROUL et al, 2016).

Essa via exerce papel fundamental na fisiologia do fígado, como especificação da linhagem, diferenciação, proliferação e adesão celular, renovação de células estaminais, transição epitelial mesenquimal, regeneração hepática e principalmente regulador da homeostase hepática (ZUCMAN-ROSSI, 2010; GUERRA, 2014; THOMPSON & MONGA, 2007).

No fígado saudável a via Wnt/β-catenina está comumente inativa, mas em condições patológicas como CHC aproximadamente 50% dos pacientes apresentam mutações nessa via, comumente no gene APC ou no próprio gene da *β-catenina*, o qual se acumula nas células tumorais (INAGAWA et al., 2002) e consequentemente ativa os genes-alvo, como os oncogenes ciclina D1 (CCND1) e c-Myc (MOON et al., 2004). Dessa forma, a sinalização é temporariamente hiperativada, sendo essencial na morfogênese hepática e disseminação do tumor (PERUGORRIA et al., 2019; LEMBERGUER et al., 2018).

**Figura 3:** Vias moleculares da hepatocarcinogênese.




**Fonte:** Adaptado de**:** Shiraha et al. (2013)

Na tentativa de mimetizar o CHC de humanos, pesquisadores estabeleceram diversas linhagens celulares, como THLE-2 e THLE-3 (PFEIFER et al., 1993); OUMS-29 (FUKAYA et al., 2001); IHH (*immortalized human hepatocyte*) (SCHIPPERS et al.,

1997); HepLL,(LI et al., 2005); HuH7 (NAKABAYASHI et al., 1982) entre outras. A linhagem HuH7.5 compreende um dos subtipos de carcinoma primário, é derivada de hepatócitos bem diferenciados, do tipo epitelial e se desenvolve em monocamadas. Foi desenvolvida em 1982 por Nakabayashi & Sato a partir da linhagem HuH7 e, segundo estudos, atua na inibição da proliferação celular pelo interferon alfa (IFN-α) e apresenta mutações nulas e pontuais no codon 220 (Y220C), é do tipo selvagem mutado para p53 e β-catenina na HuH7 (NAKABAYASHI et al., 1982; ZHAO et al., 2018).

### 1.2.2 Tratamentos

O CHC em estágio inicial é geralmente indicado o transplante de fígado, seguido da ressecção cirúrgica, ablação ou terapia transarterial, a escolha da terapia é baseada na extensão do tumor, condição de desempenho, função hepática e disponibilidade de órgãos doados, contudo, devido à falta de dadores disponíveis e às diminutas compatibilidades entre dador e doente, esta terapia torna-se útil em apenas 5% dos doentes (GUERRA, 2014). Por outro lado, para CHC avançado existem poucas opções de tratamento eficazes, apesar de várias décadas de investigação com diferentes quimioterápicos (SIM & KNOX, 2018).

A ressecção cirúrgica por hepatectomia parcial é mais indicada para pacientes que se encontram em estágio inicial, apesar de ter sido relatado resultados aceitáveis em estágios avançados de CHC (LIN et al., 2012). Entretanto, essa técnica é utilizada somente em tumores localizados, mas nunca em doença hepática associada, desde modo, a utilização desta terapia encontra-se restrita a um número reduzido de doentes, e a taxa de sobrevivência por ressecção pode ultrapassar os 50%-70% ao fim de 5 anos (GUERRA, 2014; LIN et al., 2012).

As terapias ablativas ablação por radiofrequência (ARF), ablação por microondas e alcoolização tumoral percutânea (ALC) têm pequeno potencial para cura nos tamanhos inferiores a 2 cm (estágio BCLC 0), estas terapias ablativas são mais comumente utilizadas em pacientes em que a ressecção hepática e o transplante são contraindicados por alto risco cirúrgico devido tanto à idade elevada como a presença de comorbidades clínicas (MATILLA PEÑA et al., 2012).

Já as terapias transarteriais agem na obstrução transarterial com ou sem quimioterapia regional para bloquear o suprimento de sangue ao CHC e induzir a

necrose tumoral sem influenciar significativamente o suprimento sanguíneo ao fígado, pois o fígado comprometido recebe 90% do seu suprimento sangüíneo da artéria hepática e apenas 10% da veia porta. Essas terapias incluem quimioembolização transarterial (TACE), embolização transarterial branda, quimioterapia transarterial e radioembolização transarterial. Entretanto, esses procedimentos e eficácias permanecem controversos (LIN et al., 2012).

Em relação aos fármacos, o primeiro agente testado no tratamento do hepatocarcinoma foi o antimetabólito fluorouracil. No entanto, os melhores resultados encontrados foram a partir de estudos realizados com a doxorrubicina (Doxo), antibiótico da família das antraciclinas (ANT), o qual apresentou taxas de resposta entre 10-30% (COUTINHO, 2011). Esse quimioterápico age inibindo a transcrição, síntese e replicação do DNA da célula e também interage com a enzima Topoisomerase II, produzindo radicais livres e dano celular (MOURA, 2011).

Contudo, como a taxa de resposta da doxorrubicina é baixa, a utilização dela em combinação com cisplatina, INF-α e fluorouracil (PIAF), a chamada quimioterapia sistêmica, tem se mostrado mais eficaz com taxa de resposta razoável para o tratamento do CHC não ressecável (YEO et al., 2005).

Outra droga antineoplásica utilizada no hepatocarcinoma avançado é o sorafenib, uma pequena molécula inibidora de tirosinoquinases, que atua bloqueando a sinalização de Fator de Crescimento derivado de Plaquetas (PDGF), Fator de Crescimento do Endotélio Vascular (VEGF), proteína c-kit e RAF tanto nas células tumorais quanto nas células endoteliais circundantes (COUTINHO, 2011).

Diversas outras drogas foram testadas ao logo dos anos, como por exemplo, mitoxantrona, epirrubicina, doxorrubicina liposomal, mitomicina, etoposido, irinotecano, interferon, no entanto, todas elas apresentaram mínimas taxas de resposta e nenhum benefício de sobrevida. Algumas combinações de fármacos também têm sido estudadas, como os esquemas GemOx (gencitabina e oxaliplatina), FOLFOX (fluorouracil, ácido folínico e oxaliplatina), combinação de doxorrubicina associado a sorafenibe, bevacizumabe e erlotinibe, dentre outras (SBOC, 2018; VERSLYPE et al., 2012).

Contudo, o tratamento quimioterápico sistêmico é um desafio na doença tumoral, principalmente no CHC, devido aos baixos índices de resposta dessas terapias e as múltiplas variáveis das neoplasias. Portanto, só deve ser indicado nos casos em que o paciente não é elegível para nenhuma das formas de terapia local ou

biológica isolada, ou quando houver progressão de doença após qualquer destes tratamentos (COUTINHO, 2011).

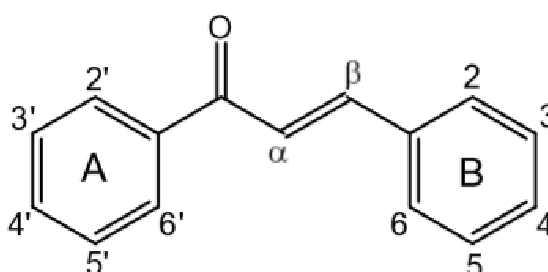
## 1.3 CHALCONAS

As chalconas, (1,3-diaryl-2-propen-1-onas), são precursores flavonóides e bioflavonóides sintetizados em plantas, sendo relevantes para a pigmentação de flores. São consideradas uma das principais classes de produtos naturais, com ampla distribuição em frutas (ex. cítricos, maçãs), vegetais (ex. tomate, batata), especiarias e chás (NOWAKOWSKA, 2006; BUKHARI et al., 2012).

São polifenóis de cadeia aberta e possuem dois anéis aromáticos ligados por um fragmento propenona de três carbonos, sendo que um anel aromático está diretamente ligado à carbonila (anel A) e o outro ao carbono β da função olefínica (anel B) **(Figura 4)** (EDDARIR et al., 2003; NOWAKOWSKA, 2006).

Essa classe de compostos apresenta inúmera atividade biológica e já foi descrita como antioxidantes, citotóxicos, antimicrobianos, antiprotozoários e anti-inflamatórios, além de apresentarem potente atividade antitumoral contra diferentes linhagens celulares (DIMMOCK et al., 1999; NI, 2004; BATOVSKA & TODOROVA, 2010; BORTOLOTTO et al., 2017).

**Figura 4:** Estrutura química da Chalcona




**Fonte:** Cordeiro (2010)

Os efeitos anti-inflamatórios das chalconas e seus precursores, de acordo com Nowakowska (2006) incluem a redução dos níveis de fator de necrose tumoral-α (TNF-α), óxido nítrico (NO), interferon-γ (IFN-γ), prostaglandinas e interleucina-6 (IL-6). Segundo estudos, este efeito se deve ao fato da inativação do fator nuclear kappa B (NF-κB), que regula mais de 400 genes envolvidos na inflamação, sobrevivência e proliferação celular, invasão, angiogênese e metástases (YADAV, 2011).

Com relação a atividade antitumoral, Mahapatra & Bharti (2015) observaram que a chalcona (1,3-diaryl-2-propen-1-onas) inibiu a cascata de sinalização JAK/STAT em células de câncer de mama (MDA-MB-231) reduzindo a fosforilação de STAT e inibindo a via Wnt, consequentemente diminuiu o crescimento e proliferação de neoplasias malignas. Ainda em câncer de mama, precursores de chalcona foram testados em linhagem MDA-MB-231 e, apresentaram efeito citotóxico, induzindo a morte celular por apoptose (FOGAÇA et al., 2017; BORTOLOTTO et al., 2017).

Makhdoumi et al. (2017), observaram em estudo *in vitro* com células HepG2 de câncer de fígado que análogos de epóxido de chalcona apresentaram efeito inibitório do crescimento celular, envolvendo mecanismos dependentes das ciclooxigenases (COX) e via da prostaglandina E2 (PGE2).

Cao et al., (2016) utilizando uma chalcona híbrida combinada com grupos trimetoxifenil e isatinil (3MCIC) observaram inibição da proliferação de células HepG2, por reduzir a ciclina B1, CDK1, p-CDK1/2 e Rb e aumentar a experssão de p53 e p21, além de ter inibido a via Wnt/β-catenina pela regulação negativa de β-catenina, c-Myc, ciclina D1 e E2F1.

Outro estudo utilizando vários compostos sintetizados, análogos à chalcona pirazólica, foram avaliados em células de carcinoma hepatocelular (Huh7), mama (MCF-7) e cólon (HCT116). Os autores verificaram que esses compostos exercerceram atividade citotóxica suprimindo significativamente a proliferação celular e ainda causaram alterações moleculares para proteínas do ciclo celular, com aumento dos níveis de p21 independente da p53 e diminuição dos níveis dos iniciadores de mitose, ciclina B1 e CDK1, com consequente parada do ciclo celular na fase G2/M seguido de morte celular por apoptose (HAWASH et al., 2017).

Ramirez-Tagle et al. (2016) também comprovaram ação citotóxica de dois compostos, 2,3,4-trimetoxi-2'-hidroxi-chalcona (CH1) e 3'-bromo-3,4-dimetoxi-chalcona (CH2), sobre células de hepatoma humano (HepG2 e Huh-7). Pelo ensaio de MTT, foram observados efeitos citotóxicos na linhagem Huh-7 com ambos as chalconas (CH1 e CH2) na concentração ($IC_{50}$ 30μM) em 24 h e na linhagem HepG2 foram utilizadas as concentrações ($IC_{50}$ 50μM) para 24 h e ($IC_{50}$ 30μM) para 48 h, além disso, foram observados eventos apoptóticos por citometria de fluxo, condensação nuclear e produção de espécies reativas de oxigênio (EROs) após 4, 8 e 24 horas.

Recentemente, Wang et al. (2019), sintetizaram uma chalcona que continha frações de indol e naftaleno (2-19), a qual apresentou atividade citotóxica contra

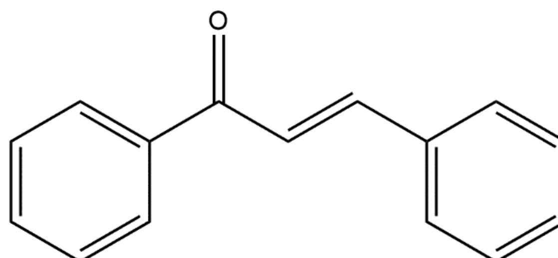
Carcinoma Hepatocelular (CHC) na concentração inibitória de 50% ($IC_{50}$ 0,65µM), além de aprisionar as células cancerígenas na fase G2/M e promover inibição da polimerização da tubulina ($IC_{50}$ 3,9µM).

### 1.3.1 *trans*-Chalcona

A chalcona pode existir em duas formas isoméricas, *cis* e *trans*, sendo a forma *trans*-chalcona (TC) considerada mais termodinamicamente estável (SILVA et al., 2015).

A TC é um produto natural que possui dois anéis aromáticos unidos por um ligante de três carbonos e, a partir da estrutura do núcleo são obtidos vários derivados conforme ilustrado na **Figura 5** (1,3-diphenyl-2-propen-1-one). Pode também ser sintetizado em laboratório pela combinação de benzaldeído (KARKHANEH, 2016; HIJOVA, 2006).

**Figura 5:** Estrutura da *trans*-Chalcona (1,3-diphenyl-2-propen-1-one).




**Fonte:** o próprio autor

A TC tem recentemente recebido um destaque singular por exercer atividade citotóxica contra muitas células tumorais, seu baixo potencial redox e lipofilicidade permite aumentar sua atividade biológica por meio de múltiplos mecanismos, incluindo ruptura do ciclo celular, inibição da angiogênese tumoral, moduladores negativos do estresse oxidativo e das respostas inflamatórias (LAMOKE et al., 2011), inibição da polimerização da tubulina, indução de apoptose além de bloqueio da via de sinalização do factor nuclear kappa B (NF-κB) (SILVA et al., 2018; MISHRA & KATARE, 2017), somado ao fato de não exibir efeitos genotóxicos sobre o grupo amino de ácidos nucleicos, que a maioria das drogas anti-câncer possuem (SILVA et al., 2015).

Segundo estudos realizados por Shen et al. (2007), o composto natural TC inibiu a proliferação de linhagens tumorais T24 e HT-1376 de câncer de bexiga por meio do bloqueio da ativação do NF-kB, pelo aumento da expressão da proteína inibitória de kappa B (I-kB) no citoplasma. Em linhagens U2OS de osteossarcoma tratadas *in vitro* com TC impediu o crescimento de asteosarco-carótidas em tempo-dependente, com consequente redução da viabilidade celular além de indução da apoptose e alteração da expressão gênica dos supressores tumorais p53 e Sp1 (SILVA et al., 2015).

Enquanto, TC testadas em linhagem celular MCF-7 de câncer mama, apresentou atividade citotóxica e genotóxica, pois causou parada do ciclo celular na fase G1, através da indução do mecanismo apoptótico e modulação de 14 genes diferentes (AIFM1, AKT1, APAF1, BAG1, BAK1, BAX, Bcl-2, BFAR, BIRC2, BIRC3, BRAF, CIDE-A, IGF1R e XIAP), através da inibição de Bcl-2 e indução de BAX e ciclina D1 após 24 horas do tratamento (BORTOLOTTO et al., 2017).

Com relação às linhagens celulares de câncer de fígado, Mishra & Katare (2017) utilizaram uma combinação sinérgica de compostos sintéticos e fitoterápicos (sorafenib, vitamina K1 e *trans*-chalcona) em linhagem celular HepG2 de CHC após intoxicação com peróxido de hidrogênio ($H_2O_2$). Os autores mostraram que houve aumento da resistência contra o estresse oxidativo gerado pelo $H_2O_2$ e que essa associação pôde proteger e restaurar os níveis normais de enzimas celulares hepáticas (AST, ALT, FAL e bilirrubina), atuando como quimiopreventivo e inibidor da hepatocarcinogênese.

Além das propriedades citotóxicas observada em diversas linhagens celulares Miranda-Sapla et al. (2019) observaram em uma análise teórica através de um estudo *in silico*, que a TC atendeu às cinco regras de Lipinski (LIPINSKI, 2004) e apresentou alta probabilidade de absorção intestinal humana, boa chance de semelhança medicamentosa e biodisponibilidade oral (LIPINSKI et al., 1997).

Além disso, as propriedades de biodisponibilidade oral e absorção, distribuição, metabolismo, excreção e toxicidade (ADMET) foram analisadas por meio do banco de dados admetSAR (CHENG et al., 2012) e o resultado demonstra que a TC não é carcinogênica (LAGUNIN et al., 2009) nem mutagênica (HANSEN et al., 2009), são fracos inibidores de hERG (*Ether-a-go-go-Related Gene*) (MARCHESE ROBINSON et al., 2011), apresentam alta solubilidade aquosa (WANG et al., 2007),

alta absorção intestinal humana (SHEN et al., 2010) e permeabilidade em células Caco-2 de adenocarcinoma de cólon humano (PHAM THE et al., 2011).

Neste contexto, o objetivo desse estudo foi investigar a ação antiproliferativa e mecanismos anti-tumorais *in vitro* da *trans*-chalcona na linhagem tumoral HuH7.5 de Carcinoma Hepatocelular humano (CHC).

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# 3 OBJETIVOS

## 3.1 OBJETIVO GERAL

- Investigar a ação antitumoral *in vitro* da *trans*-chalcona (TC) na linhagem tumoral HuH7.5 de Carcinoma Hepatocelular humano (CHC).

## 3.2 OBJETIVOS ESPECÍFICOS

- Investigar se o tratamento *in vitro* com *trans*-chalcona inibe a proliferação e sobrevivência das linhagens tumorais HuH7.5.
- Averiguar se o tratamento com TC altera a capacidade metastática das células HuH7.5.
- Determinar os mecanismos de morte envolvidos nas linhagens tumorais tratadas com TC.
- Mensurar se o tratamento com a TC altera os níveis de EROs e NO.
- Avaliar se a TC promove despolarização mitocondrial e induz formação de vacúolos autofágicos nas células HuH7.5.
- Verificar se o tratamento com TC altera o ciclo celular desta linhagem.
- Analisar por imunocitoquímica a expressão dos genes p53 e β-catenina envolvidos no CHC.

# 4 SCIENTIFIC PRODUCTION

## 4.1 ARTICLE: *Trans*-chalcone induces death by autophagy mediated by p-53 up-regulation and β-catenin down-regulation on human Hepatocellular Carcinoma HuH7.5 line.

**Article type:** Original research article

**Title:** *Trans*-chalcone induces death by autophagy mediated by p-53 up-regulation and β-catenin down-regulation on human Hepatocellular Carcinoma HuH7.5 line.

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## Highlights

- TC treatment reduces the viability of HuH7.5 tumor cells.
- The treatment with TC presents cytotoxic activity with reduction of the amount of cells.
- TC treatment increases early ROS levels and alters mitochondrial membrane potential.
- TC treatment promotes cell cycle arrest in G0/G1 and increases p-p53 expression and decreases β-catenin.
- TC treatment induces autophagy.

## Abstract

**Background:** Hepatocellular Carcinoma (HCC) is characterized by being very aggressive and presenting low response rates to available chemotherapeutic agents. The search for new natural compounds with low cost antiproliferative potential that selectively respond to liver cancer cells has been the focus of much research. In the present study, the direct action of *trans*-chalcone (TC) on cells of the human HCC HuH7.5 line was evaluated *in vitro*.

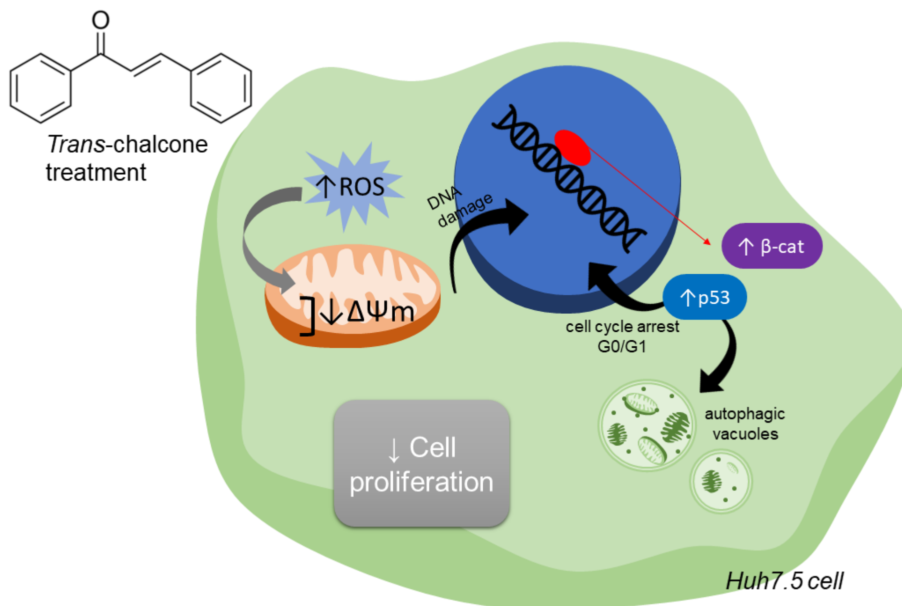
**Materials and Methods:** HuH7.5 tumor cells were treated with TC at increasing concentrations (12.5-100 μM) at 24 and 48 hours, cell viability was verified by MTT and 50% inhibitory concentration of cells ($IC_{50}$ 23.66 μM) was determined within 48 hours. Trypan blue proliferation and light microscopy, ROS production, mitochondrial depolarization and autophagy, cell cycle analysis and apoptosis by the Muse® cell analyzer and immunocytochemical markings of p-p53 and β-catenin were quantified.

**Results:** The data showed an effective dose- and time-dependent TC-cytotoxic action at low micromolar concentrations without causing toxicity to non-cancerous cells such as erythrocytes. TC-treatment caused mitochondrial membrane damage, affecting tumor suppressor gene expression (p-53) and tumor development pathway (β-catenin), inducing cell death by autophagy. In addition, TC decreased the metastatic capacity of HuH7.5, affecting the migration / invasion capacity of this cell type.

**Conclusion:** *In vitro trans*-chalcone activity in the human hepatocellular carcinoma HuH7.5 tumor cell line is shown to be a potential molecule for the development of new therapies to repair the p53 pathway and prevent overexpression of Wnt/β-catenin tumor development inducing autophagy cell death and decreasing metastatic capacity of HuH7.5 cell line.

**Key-words:** Hepatocellular Carcinoma; HuH7.5; *trans*-chalcone; EROs; Mitochondrial depolarization; Autophagy; p-53; β-catenin.

**Graphic abstract:**



# 1. Introduction

Hepatocellular carcinoma (HCC) constitute 70-85% of primary liver tumors, according to the International Agency for Research on Cancer (IARC, 2018), is the sixth most prevalent cancer worldwide and the fourth cancer-related cause of death (PAHO, 2018; GOMES et al., 2013; CAMPOS, 2017), representing a major global public health problem. According to WHO, in 2008 more than 694 thousand deaths HCC were registered in the world and in 2018 the mortality rate reached 781 thousand (AMORIM & MERCHÁN-HAMANN, 2013; GLOBOCAN, 2018).

Among the available therapies, in the early stage liver transplantation is the most efficient and indicated, followed by surgical resection, ablation or trans arterial therapies (LIN et al., 2012; SIM & KNOX, 2018), in addition to a wide range of anti-tumor drugs. However, as HCC is usually diagnosed in the late phase, systemic chemotherapy treatment is challenging, as it is very aggressive, they have low response rates, resistance to multiple drugs, several side effects and low recovery rate (COUTINHO, 2011; GOMES et al., 2013; ZHANG et al., 2019). Thus, current efforts are underway to find improved therapeutic strategies for HCC-related signaling pathways involved in the tumorigenic process.

Although cancer arises from a combination of mutations in oncogenes, developmental pathways and tumor suppressor genes in human liver cancer p53 mutations are common, which is typically highly aggressive and resistant to non-surgical therapies (XUE et al., 2007; STAIB et al., 2003; MARTINS et al., 2019). HCC also displays altered Wnt/β-catenin signaling in which more than one-third of HCC cases exhibit cytoplasmic and/or nuclear accumulation of β-catenin, a finding that correlates with poor differentiation and prognosis (LEE et al., 2006; WANDS & KIM, 2006; INAGAWA et al., 2002).

*p53* is an important gene that presents a complex role on tumorigenic suppression through repair of the tumor acting to restrict proliferation in response to DNA damage or deregulation of mitogenic oncogenes, by leading to the induction of various cell cycle checkpoints, apoptosis, autophagy, or cellular senescence (BLANDINO et al., 2019; LEE et al., 2019). Aberrant activation of this pathway gives rise to the accumulation of β-catenin in the nucleus and promotes the transcription of many oncogenes such as *c-Myc* and *CyclinD-1* (CAGATAY & OZTURK, 2002).

Thus, the development of gene therapies aimed at repairing the p53 pathway and inducing cell death would be extremely promising. In this context, chalcones (1,3-diphenyl-2-propene-1-ones) have been described as class of substances with antitumor potential (CHEN, 2001) due they are open-chain precursors for flavonoid biosynthesis and biologically classified as low molecular weight secondary metabolites (SAHU et al., 2012a,b; WONG, 1968). The chalcones structure consists of two aromatic rings joined by an unsaturated chain of three carbons and one carbonyl group. In most cases, the most stable nature of the isomer is *trans*-chalcone (TC) (1,3-diphenyl-2-propen-1-one) (EVRANOS AKSÖZ & ERTAN, 2011), it has antioxidant, anti-inflammatory properties and inhibits proliferation in different human cancer cell lines by upregulation of the tumor suppressor p53 gene (KARIME-SALES et al., 2017; KARKHANEH, 2016; SHEN et al., 2007; SILVA et al., 2015; 2018).

However, there are no studies yet in the literature with *trans*-chalcone (TC) in human Hepatocellular Carcinoma (HCC) HuH7.5 line. Therefore, this study aimed to investigate the *in vitro* antiproliferative action of this compound as well as to elucidate the possible mechanisms of death by which TC acts on this tumor cell line.

## 2. Materials and methods

### 2.1 Natural compound

Commercial *trans*-chalcone (TC), 97% pure, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA- Nero de Catogo sc-204681 – Dallas, USA, concentration 1500 µM (0,0005 mg). A stock solution of *trans*-chalcone dissolved in 1% dimethyl sulfoxide (DMSO) was prepared (GIBCO, *Invitrogen*, New York, USA). DMSO concentration did not exceed 0.01% in all experiments.

### 2.2 Tumor cell culture

HuH7.5 tumor cells grown in DMEM medium (*Dulbecco's modified Eagle's medium*, *Lifetechnologies*, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (SFB, *Lifetechnologies*), 100 U / mL penicillin and 100 µg / mL streptomycin (*Santa Cruz Biotechnologies*, Dallas, TX, USA) and kept in a 37°C incubator in a humidified 5% $CO_2$ atmosphere (*Sanyo*, Japan).

After reaching approximately 80% confluence, the cells were subcultured. For this, the cells were washed with phosphate-saline buffer (Phosphate-buffered saline, PBS, *Lifetechnologies*) and dissociated with a 0.25% Trypsin solution and 0.53 mM EDTA for 1-5 minutes. The enzyme was inactivated with DMEM medium supplemented with 10% SBF and subsequently the cells were centrifuged at 1,500 rpm for 5 minutes. Cell *pellet* was suspended in 10% DMEM medium and cells replated at the desired ratio for experimentation or maintenance. To maintain the biobank, the cells were frozen in a freezing solution (95% SFB + 5% DMSO - dimethylsulfoxide, (*Sigma*, St. Louis, MO, USA)) at a concentration of $1x10^6$ cells / mL and progressively frozen (-20°C and -80°C - Liquid Nitrogen).

## 2.3 Cell viability

To evaluate cell viability, HuH7.5 cells were deprived of SFB for 24 hours in order to synchronize the cell cycle. Subsequently, cells ($1x10^4$) were seeded in 96-well microplates and incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 24 hours for culture stabilization. The following day, cells were treated with the following *trans*-chalcone concentrations: 12.5, 25, 50 and 100 µM, whose chosen doses were based on studies already performed with TC in other tumor lines (BORTOLOTTO et al., 2017; KIM et al., 2019). Cytotoxicity was assessed 24, 48 and 72 hours after treatment by MTT metabolization (*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*), according to previous descriptions (MOSMANN, 1983; SCUDIERO et al.,1988).

To this end, the culture medium was removed and 100 µl of 0.05 mg / mL MTT solution added (Sigma, St. Louis, MO, USA). Cells were incubated at 37°C for 3 hours for MTT metabolism. After incubation, the MTT solution was removed and 100 µL of DMSO (Sigma, St. Louis, MO, USA) added for dilution of formazan crystals (30 min incubation at 37°C). Quantification of MTT metabolism was performed by spectrophotometric reading at 540 nm on the reader (*Thermo Scientific Multiskan GO*). This assay was performed in triplicate with three independent experiments. As controls, we used: Negative control, whose cells were maintained without treatment, only with supplemented medium; vehicle control, whose cells were maintained untreated, but with the presence of the *trans*-chalcone dilution vehicle (DMSO) in equal proportion in supplemented medium, and positive control, whose cells were maintained

with 25 μM ADRIBLASTINA® treatment (doxorubicin 10 mg hydrochloride - *Actavis Italy S.p.A.*, Italy, Pfizer).

From the data obtained from the cytotoxicity assay, log-regression evaluation of the $IC_{50}$ curve (50% inhibitory concentration) of the *trans*-chalcone (TC) curve was performed by the GraphPad Prisma 6 statistical program, which determined $IC_{50}$ of 23.66 μM for the 48 hour period.

### 2.4 Trypan blue exclusion cytotoxicity assessment

Initially, HuH7.5 ($3x10^4$) cells were deprived of FBS for 24 hours for cell cycle synchronization and, they were then seeded in 24-well microplates and kept in an incubator (37°C, 5% $CO_2$) for 24 hours to stabilize the culture. Subsequently, they were treated with TC-$IC_{50}$ (23.66 μM), and after 24 and 48 hours incubation with the treatment, cells were harvested, washed with PBS and dissociated with 0.25% Trypsin solution and 0.53 mM EDTA. The enzyme was inactivated with supplemented medium and the cell suspension was transferred to the same tube with storage of dead cells. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the cell pellet suspended in 100 μl PBS. To assess cell proliferation, the cell suspension was diluted in 0.4% Trypan Blue solution in a 1:1 ratio. The total number of viable and dead cells were counted by light microscopy (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) with the aid of a Neubauer camera. This assay was performed in triplicate with three independent experiments. As controls, the same conditions described in the previous item were used.

### 2.5 Quantification of HuH7.5 cell number by image microscopy

HuH7.5 cells ($1x10^6$) were seeded in a 6-well plate and kept in incubator (37°C, 5% $CO_2$) for 24 hours. Subsequently, the cells were treated with TC-$IC_{50}$ (23.66 μM) and Doxo (25 μM) and incubated for 48 hours. Photomicrographs (20x objective lens) were then performed by the EVOS FL Imaging System microscope (*Life Technologies, Thermo Fisher Scientific*) to analyze cell count after treatments.

### 2.6 *In vitro* Migration Assay

HuH7.5 cells ($1x10^6$) were seeded into 6-well microplates and incubated (37°C, 5% $CO_2$) until they reached confluence. Subsequently, a "wound" was made in the monolayer by gently passing 200 µL pipette tips into the bottom of each well. This passage led to a discontinuity in the monolayer, whose cells on its margin tended to migrate to empty spaces. Then, the cells were treated with TC-$IC_{50}$ (23.66 µM) and positive control (Doxo). Soon after, photomicrographs (20x objective lens) were performed by the EVOS microscope (*Life Technologies*) at different times (0, 12, 24 and 48 hours). Cell migration was evaluated as free area (region without cells) and this was measured with the aid of the *Image Pro Plus Program software*, and the percentage decrease of the area characterized the cell migration index. Assays were obtained in triplicates from each group tested.

## 2.7 Production of Reactive Oxygen Species

HuH7.5 cells ($1x10^4$) were seeded in 96-well microplates and incubated (37°C, 5% $CO_2$) for 24 hours for culture stabilization. They were then treated with TC-$IC_{50}$ (23.66 µM). After 4 and 48 hours, a 10 µM solution of the 2 ', 7'-dichlorofluorescein diacetate probe (H2DCFDA) (*Sigma Aldrich*, St. Louis, MO, USA) was added and incubated for 45 min in the dark. This probe is used as an indicator of ROS in cells, first detects $H_2O_2$ and hydroxyl radicals and converts them to fluorescence (VOLOBOUEVA et al., 2007). Evaluation was performed by spectrophotometric reading at 488 nm excitation and 530 nm emission on a fluorescence microplate reader (Victor X3, *PerkinElmer*, Finland). This assay was performed in triplicate with three independent experiments. As controls, we used: Negative Control, whose cells were maintained only with supplemented medium; Positive Control, whose cells were maintained with 100 µM hydrogen peroxide (*Sigma Aldrich*) to induce the production of reactive oxygen species.

## 2.8 Mitochondrial Membrane Potential Determination

Mitochondrial membrane potential (ΔΨm) analysis was performed by tetramethylrodamine-ethyl ester (TMRE) labeling (Sigma, St. Louis, MO, USA). For this purpose, HuH7.5 cells ($1x10^4$) were seeded in 96-well microplates and incubated for 24 hours. Subsequently, they were treated with $TC-IC_{50}$ (23.66 µM). One day before the first reading, 0.8 µl/well of CCCP (Carbonyl cyanide m-chloro-phenylhydrazone) was added. One hour before completing 48 hours, the CCCP (0.8 µl/well) and 25 nM TMRE solution (2µl/well) were added again and incubated for 30 min at 25° C. All wells were then washed with 200µl PBS and immediately analyzed in a fluorescence microplate reader (Victor X3, *PerkinElmer*, Finland) with excitation wavelengths (480nm) and emission (580nm). As controls, we used the same conditions described in the previous item, however as Positive Control, cells were treated with 8 µM CCCP (*Sigma*, St. Louis, MO, USA) to induce depolarization of the inner mitochondrial membrane. Fluorescence values were normalized to the total number of cells in each treatment.

## 2.9 Determination of autophagic vacuoles

For quantitation of autophage vacuoles, HuH7.5 cells ($1x10^4$ cells) were seeded in 96-well black microplate and treated with $TC-IC_{50}$ (23.66 µM) and positive control (Doxo) as described above. One hour before completing 48 hours of treatment, they were washed twice with 200 µl PBS, added 100 µl/well monodanzylcadaverine (50 µM) (*Sigma-Aldrich*, St. Louis, MO, USA) and incubated for 1 hour (37°C, 5% $CO_2$). Before reading, they were washed again with PBS and 100 µL of it added for reading. Data were obtained using a microplate fluorescence reader (Victor X3, *PerkinElmer*, Finland) with excitation wavelength (380 nm) and emission (525 nm).

## 2.10 Cell Cycle Change

HuH7.5 cells ($2x10^6$) were seeded in 6-well plate and kept in incubator (37°C, 5% $CO^2$) for 24 hours. Subsequently, cells were treated with $TC-IC_{50}$ (23.66 µM) and incubated for 24 hours. After this period, the cells were dissociated with 300 µl of 0.25% Trypsin solution and 0.53 mM EDTA for 2 min in a greenhouse (37°C, 5% $CO_2$). The cell suspension was centrifuged at 1,000 rpm/5 min. Subsequently, the supernatant was discarded and 300 µl PBS was added, homogenized and 100 µl of this solution

transferred to 1.5 ml eppendorf and 50 µl of the solution containing 0.1% citrate and 50 µg/ml propidium iodide (PI) were added and then incubated in a freezer for 20 min. The analysis was then started by the Muse® Cell Analyzer and 2,500 events were collected for population evaluation in G0/G1, S, G2/M. Analysis of the results was performed using Muse Cell Analyzer™ software.

### 2.11 Immunocytochemical detection of p-p53 and β-catenin

Immunocytochemical analyzes were performed on coverslip-adherent HuH7.5 cells using the labeled streptavidin biotin method using LSAB KIT (DAKO Japan, Kyoto, Japan). The slides were incubated with 10% Triton X-100 solution for 1 hour, washed 3 times in PBS and treated for 40 min at room temperature with 10% BSA. In addition, coverslips were incubated overnight at 4°C with primary antibodies β-catenin (1: 500) and p-p53 (1: 300) (*Santa Cruz Biotechnology*). After secondary antibody treatment (2 hours, room temperature), horseradish peroxidase (*HRP*) activity was visualized by treatment with $H_2O_2$ and 3,3'-diaminobenzidine (DAB) for 5 min. Finally, the sections were weakly counterstained with Harry's hematoxylin (*Merck*). For each case, negative controls were performed omitting the primary antibody. The intensity and localization of immunoreactivities against the primary antibody used were examined on all slides using a photomicroscope (Olympus BX41, *Olympus Optical Co.*, Ltd., Tokyo, Japan). For the study of image analysis, photomicroscopic color slides from representative areas (40x objective lens) were digitally acquired. To determine a semi-quantitative score, images were evaluated using the Image J software color deconvolution tool (NIH, USA). The pixels were categorized as described previously by Chaterjee et al. (2013) as high positive (3+), positive (2+), low positive (1+) and negative (0).

### 2.12 Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Invitrogen, Eugene, USA), a calcium-dependent phospholipid binding protein used as an

apoptosis marker. The HuH7.5 cell ($2x10^6$) were submitted to a 48 h with TC-$IC_{50}$. After this period, the cells were dissociated with 300 µl of 0.25% Trypsin solution and 0.53 mM EDTA for 2 min in a BOD incubator (37°C, 5% $CO_2$). The cell suspension was centrifuged at 1,000 rpm/5 min. Subsequently, the supernatant was discarded and 300 µl PBS was added, homogenized and 100 µl of this solution transferred to 1.5 ml eppendorf and for staining 50 µl of Annexin V FITC solution (Guava Nexin®, BD, Pharmingen, USA) at room temperature (25 ℃) were added and then incubated in a freezer for 20 min. Data acquisition was performed using the Muse® Cell Analyzer (Merck Milipore). Analysis of the results was performed using Muse Cell Analyzer™ software. For a comparison, we normalized the fluorescent values obtained.

### 2.13 Statistical analysis

Data were expressed as mean ± standard error of the mean. Three independent experiments were performed, each in triplicate. Data were analyzed using the GraphPad Prism 6.01 statistical program (GraphPad Software, Inc., San Diego California, USA). Significant differences between groups were determined by One-way ANOVA, followed by Tukey's test for multiple comparisons. Differences were considered statistically significant when $p \leq 0.05$.

## 3. RESULTS

### 3.1 TC has antiproliferative effect on cell HuH7.5 and reduces metastatic capacity

In order to investigate the cytotoxic effect of TC against HCC HuH7.5 cells, the MTT assay was performed. We observed that cell viability of the HuH7.5 line in the groups treated with TC (12.5; 25; 50 and 100 µM) decreased significantly ($p \leq 0.0001$) at all times tested when compared to the control (**Fig. 1A, B**). At 24 h, higher doses of TC (25; 50 and 100 µM) were more effective as antiproliferative agent on tumor cells than the positive control (Doxo 25 µM) ($p \leq 0.0001$) and the concentration 100 µM reduced cell viability by 65.2% when compared to the control, being the most effective of the tested concentrations (**Fig. 1A**). TC concentrations (25 and 50 µM) did not differ from each other, reducing cell proliferation by 39.8 and 45.6% respectively **(Fig. 1A).**

When evaluated the effect of TC at 48h of treatment, we verified a dose and time dependent effect when compared to 24h, significantly reducing cell viability by 22.6, 64.4, 77.7, 88.6% when treated with TC-concentrations of 12.5, 25, 50 and 100 μM, respectively (**Fig. 1B, C**).

At 48 hours, there was no significant difference between TC treatment at concentrations 50 and 100 μM and positive control, Doxo 25 μM **(Fig. 1B)**. Subsequently, the $IC_{50}$ (50% inhibitory concentration) of TC on HuH7.5 cells was determined. The results showed the $IC_{50}$ 53 μM (± 0.04) and 23.66 μM (± 0.02) for 24h and 48h, respectively. Thus, as the results were time dependent, we proceeded with the other experiments using the concentration 23.66 μM at the time of 48 hours **(Fig. 1C)**, and also because the replicative period of liver tumor cells is 14 to 48 hours and mitotic peak at 48 hours (JESUS et al., 2000). In addition, even with regard to cytotoxicity properties, it is worth mentioning that the TC-concentrations tested had no hemolytic effect on erythrocytes (data not shown).

In order to confirm the results found in the MTT assay, we counted of the number of viable cells by trypan blue exclusion method. After 48 hours of HuH7.5 cell TC-$IC_{50}$ treatment, we found that the TC treatment showed a cytotoxic effect reducing 51.3% the amount of viable cells when compared to the control ($p \leq 0.0001$) and as illustrated by the representative image **(Fig. 1 D,E)**.

Knowing that the ability of tissue migration and invasion is a hallmark of malignant tumor cells, we also analyzed if the metastatic capacity of the HuH7.5 cell was altered by TC treatment, by the cell migration assay. The results showed that the control group cells present a wound closure capacity since 12h of observation, reaching the maximum closing of 78.7% from 24h **(Fig 2 A,B).** The TC-$IC_{50}$ inhibited significantly this metastatic capacity of HuH7.5. After 12 h, only 1% of wound closure occurs, and at 24h and 48h, the distance reduction was significantly smaller than the control, reaching only 39.5% and 26.6% of wound closure, respectively **(Fig 2 A,B).**

According to these findings, the *in vitro* TC treatment showed direct cytotoxic effect on HCC HuH7.5 reducing the main characteristics of cancer cells, cell proliferation and the ability of migration and invasion of this cell line.

### 3.2 TC alters early ROS levels and promotes mitochondrial membrane depolarization

As *in vitro* treatment with TC has shown to have a direct effect on the HuH7.5 line, we sought to investigate how this compound acts on these tumor cells. Initially it was evaluated whether the compound alters the levels of ROS. HuH7.5 cell TC-$IC_{50}$ treatment resulted in an increase in initial ROS production. (4 hours of treatment) compared to the control group ($p \leq 0.005$) **(Fig. 3A)**. After 48 hours of treatment, although there is a tendency for ROS production in treated cells, it was not significant when compared the control group **(Fig. 3B)**.

It is known that the significant increase of ROS can result in direct damage to mitochondria leading to changes in the functioning of this organelle. Therefore, we evaluated the integrity of mitochondrial functioning by analyzing mitochondrial membrane potential by TMRE staining which complexes with active mitochondria, since the significant loss of ($\Delta\Psi m$) renders cells exhausted with subsequent death. It was found that after 48 hours of the HuH7.5 cell TC-$IC_{50}$ treatment, there was a reduction in the fluorescence intensity of the TMRE compared to the control group in HuH7.5 ($p \leq 0.001$) **(Fig. 3C)**, indicating loss of mitochondrial potential membrane similar to positive control (CCCP treatment).

### 3.5 TC increases tumor genes expression p-p53 and decreases β-catenin in HuH7.5 cells

Additionally, we determined the distribution of HuH7.5 cells treated with TC-$IC_{50}$ at different stages of the cell cycle. Our results show that TC treatment results in a slight change in the percentage of cells between phases of the cell cycle. The HuH7.5 cell TC-$IC_{50}$ treatment increased the percentage of cells in G0/G1 by 7% ($p \leq 0.005$), decreased the percentage of cells in phase S by 5.2% when compared to untreated cells ($p \leq 0.05$). There was no difference in the percentage of cells in the G2/M phase. These results, together, show that although slight changes have occurred in the cell cycle the main mechanism of action of TC is not related to cell cycle disruption at any stage other than this one **(Fig. 4 A,B)**.

To verify the expression of genes involved in tumorigenesis, immunocytochemical markings of p-p53 and β-catenin were performed on HuH7.5 cells. P53 is a tumor suppressor gene which is mutated at codon 220 (Y220C) in HuH7-derived cell line, being crucial for oncogenic cell activity (NAKABAYASHI et al., 1982; ZHAO et al., 2018). Our results showed that control cells did not express p-p53

labeling, however, TC-$IC_{50}$ treatment restored p-p53 expression by significantly increasing the nuclear and cytoplasmic labeling of the gene ($p \leq 0.01$) **(Fig. 4C),** reestablishing the control phenotype in which DNA binding capacity to p-53 was strongly suppressed. We also evaluated the expression of the Wnt / β-catenin pathway, which is mutated in HuH7.5 resulting in the activation of oncogenes such as cyclin D1 and c-Myc. The results show that there is a strong β-catenin labeling in untreated cells, which is suppressed by TC-$IC_{50}$ treatment ($p \leq 0.01$) **(Fig. 4E).**

These data suggest that HuH7.5 cell TC-$IC_{50}$ treatment is able to effectively and significantly reverse activated/inactivated genes involved in the tumor mechanism.

### 3.6 TC induces autophagy process resulting in cell death

Still investigating the possible mechanisms of death exerted by TC against the HuH7.5 line, apoptosis and autophagy analysis was performed on these cells. Autophagy generally functions as a cell survival mechanism but it has been identified the autophagic response to p53 activation as important for p53-mediated cell death.

By marking autophagic vacuoles by MDC it was observed that after 48 hours of TC-$IC_{50}$ treatment, there was an increase in autophagic vacuole formation compared to untreated control ($p \leq 0.01$) **(Fig. 5A)**. When phosphatidylserine (PS) expression was evaluated as apoptotic marker we observed that the TC-$IC_{50}$ treatment did not alter PS expression after 48h of treatment **(Fig. 5B).**

## 4. DISCUSSION

Given the lack of effective therapies for human hepatocellular carcinoma (HCC), especially in the advanced stages of the disease, it is particularly important to find improved therapeutic strategies for HCC. Therefore, the discovery and development of new drugs based on natural products have been the focus of many researches (RAYAN et al., 2017). In this context, trans-chalcone (TC), a natural product that has already confirmed its antiproliferative properties in other types of tumor cells, emerges as a potential compound in the treatment of liver tumor cell line.

In this sense, our study sought to elucidate new perspectives of the TC-mechanism of action against *in vitro* human HCC HuH7.5 cell line. Data showed an effective TC-cytotoxic action in a dose and time-dependent manner at low micromolar concentrations without causing toxicity to non-cancerous cells such as erythrocytes. TC treatment caused mitochondrial membrane damage, affecting the expression of tumor suppressor gene (p-53) and tumor developmental pathway (β-catenin) inducing cell death by autophagy. In addition, TC decreased HuH7.5 metastatic capacity affecting the migration/invasion capacity of this cell type.

The anticancer activity of most natural products often act via regulating immune function, inducing apoptosis or autophagy, or inhibiting cell proliferation (reviewed in RAYAN et al., 2017). About the TC antitumor activity, there are reports showing the antiproliferative activity of TC against several different human tumor cell lines, such as T24 and HT-1376 bladder cancer (SHEN et al., 2007), endometrial carcinoma Ishigawa lineage, and MCF-7 and MDA-MD-231 breast cancer (BORTOLOTTO et al., 2017; MATEEVA et al., 2017), U2OS and SJSA-1 osteosarcoma cells, HCT-116 colon carcinoma, FaDu epitelial cell line (SILVA et al., 2015; 2018) and murine fibroblasts (3T3) (BORTOLOTTO et al., 2017). However, there are no reports in the literature about the effect of TC on HuH7.5 tumor lines.

This antiproliferative effect of TC was mainly demonstrated by inhibition of the nuclear factor kappa B (Nf-κB) (SHEN et al., 2007); upregulation of the tumor suppressor p53 gene (SILVA et al., 2015; 2018) resulting in apoptosis death by mitochondrial apoptotic pathway (SHEN et al., 2007; BORTOLOTTO et al., 2017; SILVA et al., 2015; 2018).

While containing many mutations and insertion or deletion of bases in the cell genome, it is worthy to note that the Huh7 cells have somatic mutations such as the p53 cell cycle (ZHAO et al. 2018). Cagatay & Ozturk (2002) also showed a perfect correlation between p53 gene mutation and aberrant accumulation of β-catenin protein on HuH7 cell line.

In this context, our results demonstrated repairing in the p53 pathway where the HuH7.5 TC-$IC_{50}$ treatment increased the levels of p-p53 corroborating to Silva et al. (2015; 2018) where showed that the rescue of p53 activity in mutant p53 cancer cells induced regression of osteosarcoma cells (U2OS, SJSA-1), colon carcinoma (HCT-116) and FaDu epitelial cell line. In addition, we observed a strong β-catenin suppression on TC-$IC_{50}$ treatment, reporting for the first time, the action of TC on

Wnt/β-catenin pathway, which is generally overactivated under pathological conditions such as HCC (INAGAWA et al., 2002).

Interestingly, our data showed that HuH7.5 treatment with TC-$IC_{50}$ did not induce apoptosis, a cell death often associated with increased p53 expression over the period evaluated (CHARNI et al., 2014). However, the upregulation of p53 and Wnt/β-catenin pathway has already been shown to be associated with autophagy, a process known as cellular recycling through the formation of autophagic vacuoles that allow programmed protein degradation and organelle turnover that contribute to the maintenance of cellular homeostasis (RABINOWITZ & WHITE, 2008; MOLDER et al., 2018; SHANG et al., 2017).

Regarding to autophagy, its role in hepatocellular carcinoma is still controversial, as interference with the autophagic mechanism may promote or interrupt tumorigenesis (LIU et al., 2017; MRAKOVCIC & FRÖHLICH, 2018; CORDANI et al., 2016). Sun et al. (2013) showed that the role of autophagy in the occurrence and development of HCC is dependent on the context of liver cells. During dysplastic phase in hepatocytes, basal autophagy acts as a suppressive tumor by removing newly damaged mitochondria and mutated cells and thus maintaining genomic stability. However, once a tumor is established, unbalanced autophagy will contribute to HCC cell survival under various stress conditions and in turn promotes tumor growth (SUN et al., 2013)

Although autophagy is this physiological process with an inconclusive role in liver cancer, this phenomenon may be part of the events that result in autophagic cell death, type II programmed cell death, a form of nonapoptotic cell death mechanism (SCARLATTI et al., 2009). Recently, Martins et al. (2019) showed that the autophagy process, once it is associated with parallel damage in mitochondria and lysosomes is an efficient way to induce cell death by autophagy. Our data corroborate to this work showing that HuH7.5 TC-$IC_{50}$ treatment caused early production of ROS and mitochondrial damage trough the mitochondrial membrane depolarization resulting in high and efficiently cell death confirmed by cytotoxic effect on trypan blue assay. Besides that, the TC treatment does not cause arrest at any specific phase of the cell cycle.

The mitochondrial depolarization caused by the TC treatment resulted in loss of organelle integrity. Mitochondria are known to be hyperpolarized in tumors and to modulate various biological functions, such as proliferation, differentiation, invasion,

and metastasis (ZHANG et al., 2019; MICHELAKIS et al., 2007). Cancer metastasis is a complex process of cell spreading which can be divided into migration, invasion, intravasation, survival in the circulation, extravasation, and metastatic colonization (HANAHAN & WEINBERG, 2000; MINA & SLEDGE, 2011) being the migration and invasion the crucial steps for successful metastasis (HARLOZINSKA, 2005). Our data suggest that TC-$IC_{50}$ treatment affected the ability of migration and invasion of HuH7.5 cells and may be a promising candidate for anti-metastasis use.

## 5. CONCLUSION

In conclusion, this work provided new insights of the *in vitro* TC-activity in human hepatocellular carcinoma HuH7.5 tumor cell line, as a potential molecule for the development of new therapies to repair the p53 pathway and prevent the overexpression of Wnt/β-catenin tumor development pathway, inducing autophagy cell death and decreasing the metastatic capacity of HuH7.5 cell line.

Taken together, our findings indicate that TC is a molecule for further studies of anti-metastasis and antitumor activities in human hepatocellular carcinoma and can become a lead molecule for the design of new prototypes of antitumor compounds more potent and selective.

**Abbreviations:**

HCC: Hepatocellular Carcinoma; TC: *trans*-chalcone; DMSO: dimethyl sulfoxide; DMEM: Dulbecco´s modified Eagle´s medium; SBF: fetal bovine serum; PBS: Phosphate-buffered saline; MTT: dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; H2DCFDA: 2 ', 7'-dichlorofluorescein diacetate; NO: nitric oxide; ROS: reactive oxygen species; TMRE: tetramethylrodamine-ethyl ester; CCCP: Carbonyl cyanide m-chloro-phenylhydrazone.

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**Availability of data and materials**

All data generated or analyzed during this study are indicated in this article and its supplemental data files.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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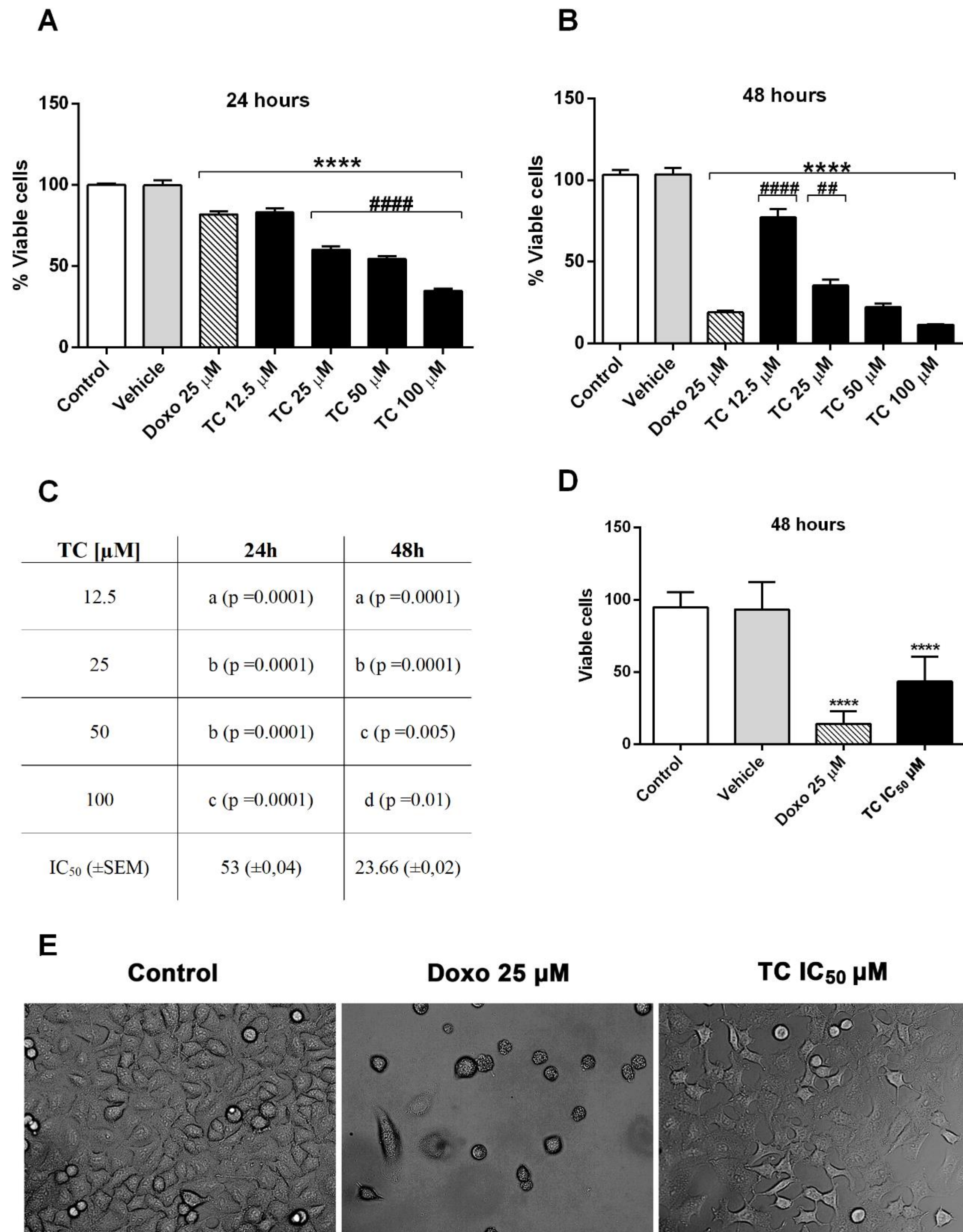



| TC [µM] | 24h | 48h |
|---|---|---|
| 12.5 | a (p =0.0001) | a (p =0.0001) |
| 25 | b (p =0.0001) | b (p =0.0001) |
| 50 | b (p =0.0001) | c (p =0.005) |
| 100 | c (p =0.0001) | d (p =0.01) |
| $IC_{50}$ (±SEM) | 53 (±0,04) | 23.66 (±0,02) |

**Figure 1:** Analysis of cell viability in HCC HuH7.5 tumor line. HuH7.5 cells were treated with TC at concentrations of 12.5; 25; 50; 100 µM for 24 hours (A) and 48 hours (B) analyzed by MTT viability assay, detailed statistical analysis between the treatments and determination of 50% inhibitory concentration of *trans*-chalcone (TC-$IC_{50}$) (C), trypan blue counting at 48 hours (D) and quantification by image microscopy (E). Control (DMEM medium), Vehicle (0.01% DMSO) and 25 µM Doxorubicin (positive control). The values represent the mean ± SEM of three independent experiments performed in triplicate. One-way ANOVA statistical method

was used followed by Tukey test. ****Significant difference from control ($p \leq 0.0001$). ##Significant difference from positive control ($p \leq 0.01$) ####($p \leq 0.0001$). + Significant difference from TC 100uM ($p \leq 0.0001$).

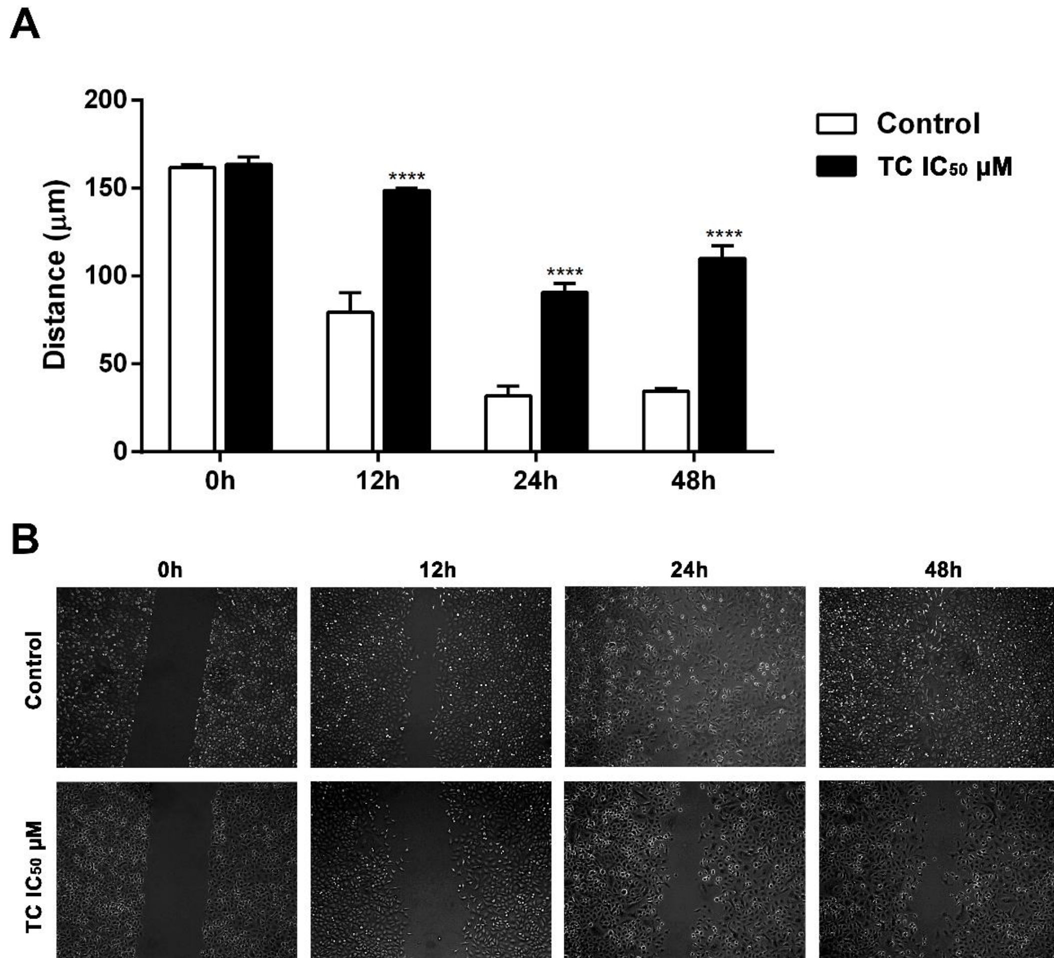



**Figure 2:** Treatment with TC-$IC_{50}$ inhibited HuH7.5 cell migration. A scratch assay was performed to observe the effect of TC on Huh7.5 cell migration over time (0, 12, 24, and 48h) (A) and the distance from cell free area after treatment with TC-$IC_{50}$ (23.66 µM) (B). The graph represents values expressed as mean ± SEM ****($p \leq 0.0001$) vs. Control.

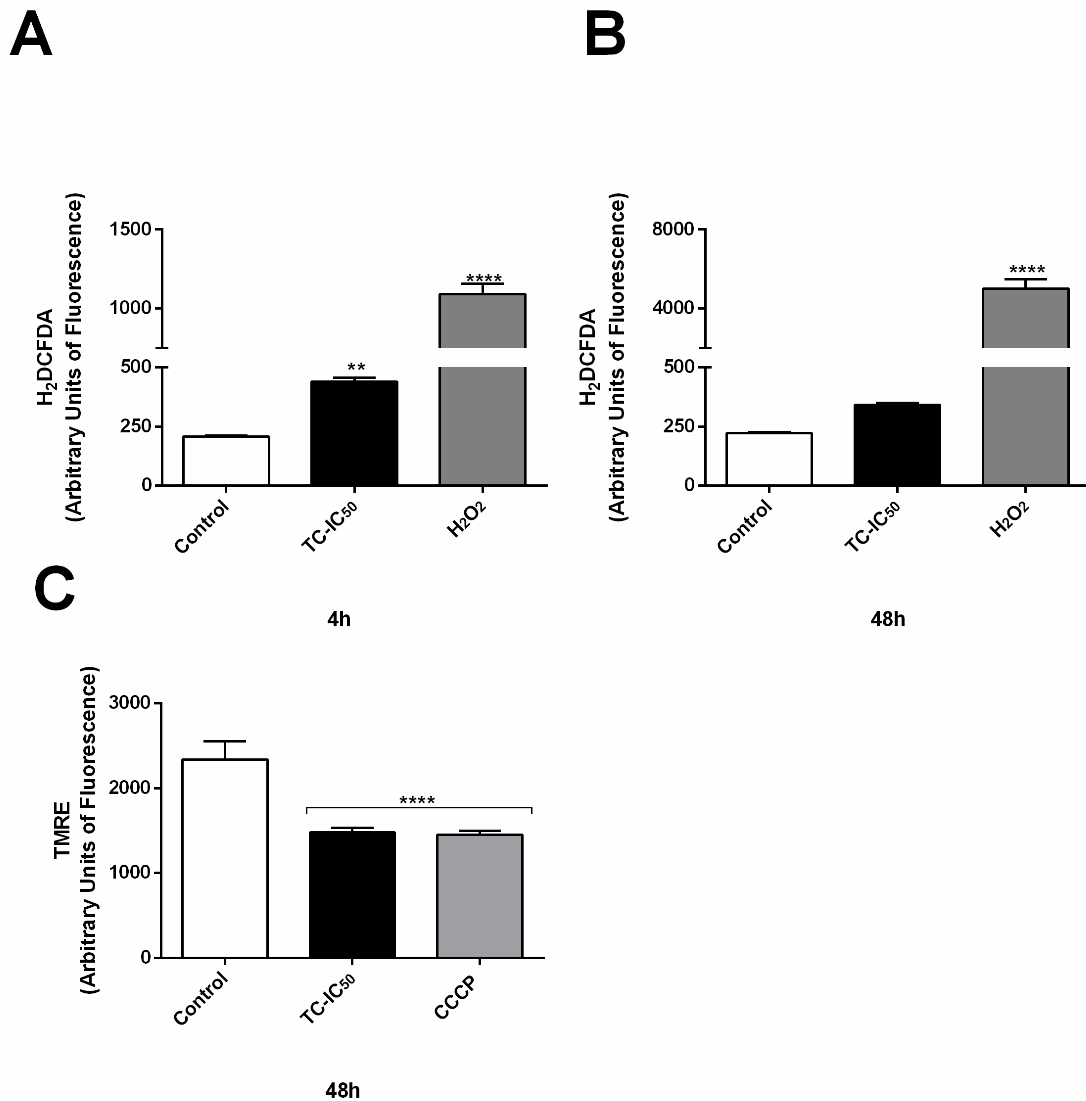



**Figure 3:** Production of reactive oxygen species and mitochondrial depolarization produced by the HuH7.5 cells. The cells were treated with TC-$IC_{50}$ (23.66 μM) and analyzed after 4 hours (A) and 48 hours (B) by the H2DCFDA probe for analysis of reactive oxygen species levels after 48 hours, TMRE probe for verification of mitochondrial membrane potential (C). $H_2O_2$ and CCCP were used as positive controls. The values represent the mean ± SEM of three different experiments performed in triplicate. **Significant difference from control ($p \leq 0.01$), ***($p \leq 0.001$) and ****($p \leq 0.0001$).

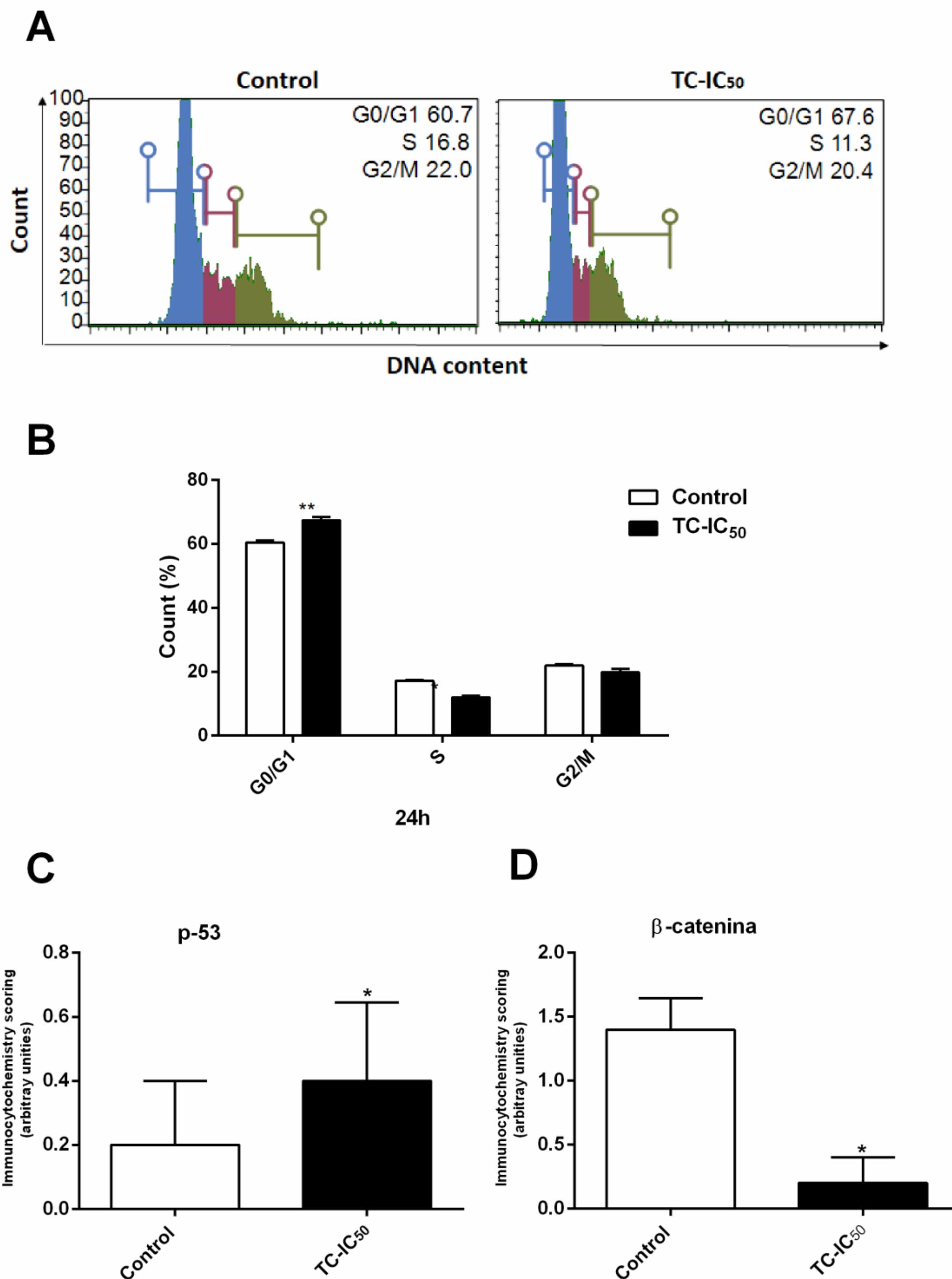
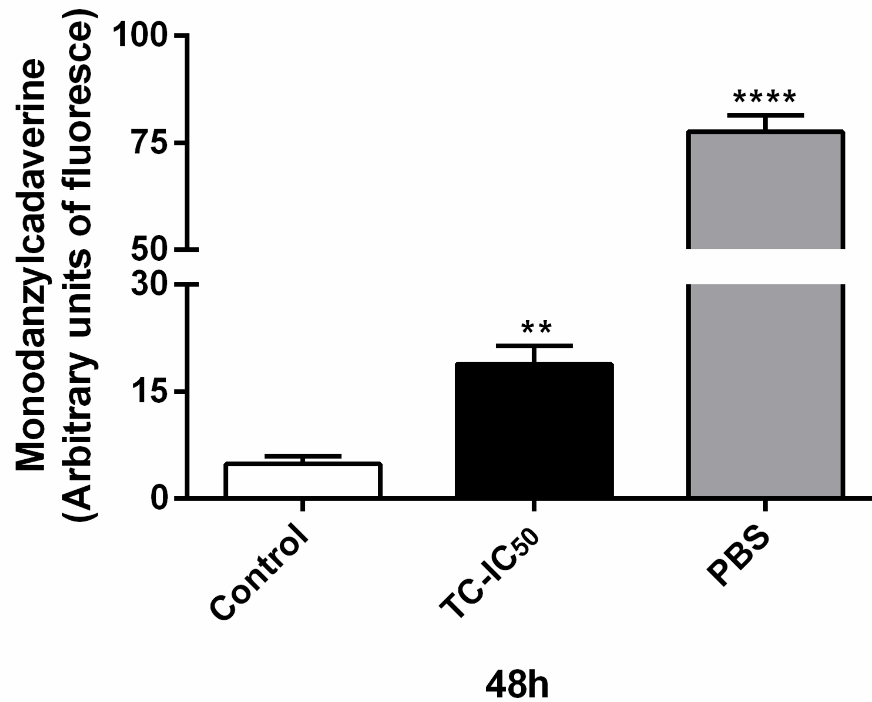



**Figure 4:** Induction of cell cycle arrest and expression of genes involved in HCC. Induction of cell cycle arrest for 24 hours (A) e immunocytochemical analysis of β-catenin, c-Myc and p-p53 proteins (B) in the HuH7.5 cells treated with TC-$IC_{50}$ (23.66 μM). Immunocytochemistry scoring were classified as high positive (3+), positive (2+), low positive (1+) and negative (0). *Significant difference from control ($p \leq 0.01$) and ****($p \leq 0.0001$). #Significant difference from positive control ($p \leq 0.01$), ## ($p \leq 0.001$) and #### ( $p \leq 0.0001$).

A




B

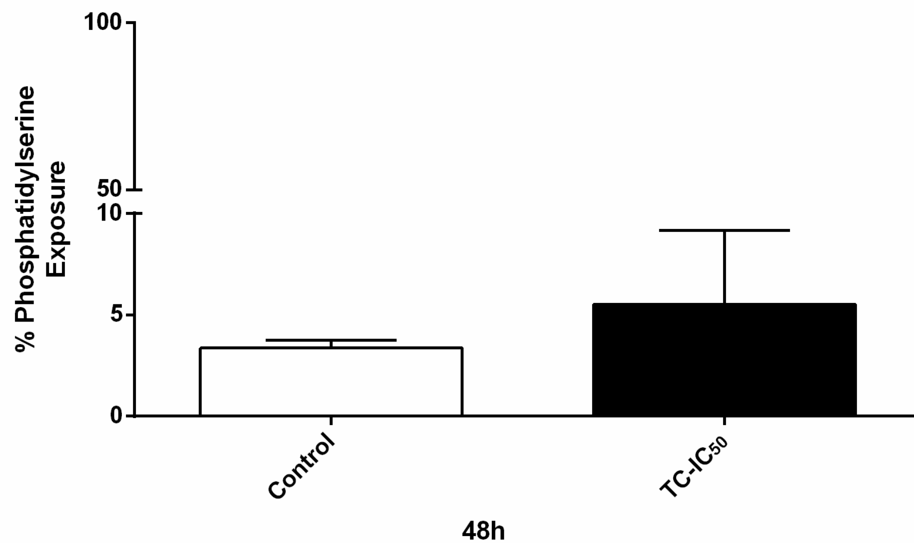



**Figure 5:** Production of autophage vacuoles and phosphatidylserine exposure of the CT-treated HuH7.5 tumor line cell for 48 hours. Cells were treated with TC-$IC_{50}$ (23.66 μM), PBS was used as positive control. The values represent the mean ± SEM of three different experiments performed in triplicate. **Significant difference from control ($p \leq 0.01$) and ****($p \leq 0.0001$).