

# Cytotoxic activity related to survivin mRNA levels by *Combretum quadrangulare* Kurz extract against liver and breast cancer cells

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## ABSTRACT

Liver cancer and breast cancer have high death rates and tend to increase rapidly in recent years in Vietnam and over the world. Currently, the study of bioactive compounds isolated from plants for killing cancer cells is of interest. The study aims to identify the cytotoxicity using MTT assay of *Combretum quadrangulare* Kurz fractions against MCF-7 cells. The RT-qPCR method was used to determine the *survivin* mRNA levels in P12 fraction-treated HepG2 and MCF-7 in comparison to DMSO-treated cells. The results showed that the P12 fraction with IC<sub>50</sub> of 20.9 ± 0.8 μg/mL was the best cytotoxicity on MCF-7 cells amongst P fractions. P12 fraction exhibited the apoptotic effect on HepG2 and MCF-7 cells by down-regulating *survivin* expression levels. Thus, the P12 fraction showed promising potential for further research about *in vivo* fractions assessment or bioactive compounds isolation.

## 1. Introduction

Every year, there are about nine to ten million people suffering from cancer globally, and half of them die from this disease (Ghoncheh, Pournamdar, & Salehiniya, 2016). In 2020, there were about 2.3 million women detected with breast cancer and 685,000 deaths globally, making it the global most common cancer (Printz, 2021). In addition, worldwide estimates had 905,677 new liver cancer cases, and 830,180 related deaths were recorded in 2020 (Bray et al., 2018; Sung et al., 2021). According to 2020 statistics, Globocan (Global cancer observatory) showed that Vietnam was ranked as a country with the highest rate of liver cancer in the world (Sung et al., 2021) with 26,418 new cases (14.5%) and 21,555 (11.8%) new cases for breast cancer. In 2020, there were 25,272 and 9,345 deaths caused by liver cancer and breast cancer, respectively. Because of the increasing incidence of new cases and deaths, breast cancer and liver cancer are worthy of attention in Vietnam as well as in the world.

*Combretum quadrangulare* is a small tree of the *Combretaceae* family and has grown popular in Vietnam, Cambodia, Laos, Myanmar, and Thailand. *C. quadrangulare* was reported with a number of uses such as antibacterial (Nantachit, Tuchinda, Khantawa, & Roongjang, 2015; Somanabandhu, Wungchinda, & Wiwat, 1980), anti-HIV (Narayan et al., 2011), anticancer ability (Eloff, Katerere, & McGaw, 2008; Nopsiri, Chansakaow, Putiyanan, Natakankitkul, & Santiarworn, 2014), and liver protection activity (Banskota, Tezuka, Adnyana, et al., 2000). The

extracts, as well as compounds extracted from roots, flowers, and leaves of *C. quadrangulare*, have been shown toxic effects on many types of cancer cells.

Based on the premise studies, *C. quadrangulare* has many potentials in finding extracts containing ingredients with high biological activity (Roy, Singh, Jash, Sarkar, & Gorai, 2014). Regarding cytotoxic activity, Methyl quadrangulare B and D of *C. quadrangulare* expressed toxicity on murine colon 26-L5 carcinoma cells with ED<sub>50</sub> value 9.54 and 5.42μM, respectively (Banskota et al., 1998; Banskota, Tezuka, Tran, et al., 2000a). Combretin, a steroidal alkaloid isolated from the seeds of *C. quadrangulare* Kurz, showed moderate anticancer activities against human hepatocarcinoma (HepG2) HB-8065 and caucasian colon adenocarcinoma (CaCO<sub>2</sub>) HTB-39 (Nantachit, Sirilun, Nobsathian, & Rungjang, 2017).

Cancer cells can be killed by two main mechanisms containing apoptosis and necrosis. Apoptosis is a natural process that ensures the growth and development of cells under control. In contrast to cell necrosis, apoptosis with the apoptotic bodies is always phagocytosed without causing the inflammatory process. Thus, the strategies to induce apoptosis in cancer cells are of great significance.

However, apoptosis is prevented, causing the immortalization of cancer cells while also leading to the maintenance of mutations in the body. Inhibition of apoptosis leads to tumorigenesis, allowing the accumulation of mutations that trigger the transformation of normal tissue cells (Vazquez, Bond, Levine, & Bond, 2008). The key regulators of apoptosis are Bcl-2 and inhibitors of apoptosis (IAP) families proteins (Kang & Reynolds, 2009). Cancer cells can turn off apoptosis by interfering the balance of pro-apoptotic and anti-apoptotic proteins, decreasing function of the caspase enzymes; attenuation of the death receptors signaling.

Survivin, also known as BIRC5 (baculoviral inhibitor of apoptosis repeat-containing 5), is a protein of inhibitor of apoptosis (IAP) family. Survivin was first described by Ambrosini in 1997 (Ambrosini, Adida, & Altieri, 1997). Survivin was proven to have an important role in inhibiting cell apoptosis by down-regulation of pro-apoptotic proteins. A previous study showed that survivin binds specifically to the terminals of caspase-3 and caspase-7, resulting in decreasing expression of these caspases (Tamm et al., 1998). Survivin can directly bind to caspase-9, inhibits the activity of this caspase, and leads to inhibiting apoptosis (Chandele, Prasad, Jagtap, Shukla, & Shastri, 2004). In addition, survivin inhibited activation of procaspase-8, Bid, procaspase-9, and procaspase-3 in survivin-knockdown hepatocytes, increased sensitivity to Fas receptor of extrinsic apoptosis pathway (Conway et al., 2002). The studies determined the characteristics of survivin in the inhibition of apoptosis. A number of studies have demonstrated that inhibition of survivin gene expression leads to apoptosis in cancer cells (Conway et al., 2002; Chandele et al., 2004; Li et al., 1998; Liu et al., 2008).

In cancer research, herbal plant extracts are valued for their abilities to provoke cytotoxicity through apoptosis. At present, there are not many studies proving that the extracts from *Combretum quadrangulare* have toxic effects on breast cancer cells and further mechanisms of cytotoxic activity on cancer cells. In addition, the premise study (Nguyen et al., 2021) showed the good cytotoxic ability of the extracts against hepatocellular carcinoma. Thus, this study was carried out to investigate the possibility that *C. quadrangulare* fractions can be cytotoxic breast cancer cells. And further investigation of the P12 fraction effect on the apoptosis of HepG2 and MCF-7 cancer cell by way of decreasing expression levels of IAP genes such as *survivin* gene.

## 2. Materials and methods

### 2.1. Materials

P fractions, P1-P14, and PAX1 fractions, from *C. quadrangulare* Kurz were obtained from the previous research (Nguyen et al., 2021). In brief, fraction HEA (Hexan: Ethyl acetate, from crude extract) was subjected to silica gel column chromatography, using an isocratic mobile phase containing n-hexane: EtOAc: acetone (5:1:1) system to collect the P fractions. Liver cancer cell line HepG2 and breast cancer cell line MCF-7 were achieved from Ho Chi Minh City Biotechnology Center.

### 2.2. Methods

#### 2.2.1. Cells viability assay

**Cell culture.** Two cancer cell lines were seeded in a complete medium of DMEM high glucose (Hyclone) with 10% fetal bovine serum (FBS, Hyclone) supplementation, 100µg/ml Penicillin/Streptomycin antibiotics (Hyclone) and incubated at 37°C and 5% CO<sub>2</sub>. The cells, reaching the adhesive density up to 80%, will be subcultured using 0.25% Trypsin-EDTA. Cells will be diluted to a density of 10<sup>5</sup> cells/ml in a complete medium. 100µl of diluted cells will be added to each well in a 96-well plate (Corning), then incubate the cell plate in an incubator (37°C and 5% CO<sub>2</sub>) for 24 hours.

**MTT assay.** MTT assay was used to evaluate the toxic activity of extracts and fractions on MCF-7 cells. The extracts were diluted in culture medium at concentrations of 100µg/ml; 50µg/ml; 25µg/ml; 12.5µg/ml; 6.25µg/ml; 3.125µg/ml and 0µg/ml. In which, equivalent DMSO concentration is 1%; 0.5%; 0.25%; 0.125%; 0.0625%; 0.03125% and 0%. The negative control was DMSO, with seven concentrations corresponding to DMSO concentration in the extracts. Positive control was Doxorubicin (DOX, Kabi, India). The wells that contained the concentrations of Doxorubicin in the culture medium without cultured cells were used as Blank. The cells were incubated at 37°C and 5% CO<sub>2</sub> for 72 hours. Morphological changes were obtained after every 24 hours. The absorbance of samples was read at 570nm by an ELISA reader. An experiment was conducted in triplicate. The cytotoxicity of the fractions was determined by IC<sub>50</sub> using GraphPad Prism software. Cell death rate (Inhibition, I) at the concentrations of fractions were calculated in Excel according to the formula:

$$I\% = 100 - 100 * (A_{\text{Extract}} - A_{\text{Blank}}) / (A_{\text{DMSO}} - A_{\text{Blank}}) \quad (1)$$

$A_{\text{Extract}}$ : Absorbance of extract at 570nm wavelength.

$A_{\text{DMSO}}$ : Absorbance of samples containing DMSO at 570nm wavelength.

$A_{\text{Blank}}$ : Absorbance of Blank at 570nm wavelength.

#### 2.2.2. Effect of *C. quadrangulare* P12 fraction on survivin mRNA levels

**Acquisition of treated cells.** 5×10<sup>5</sup> HepG2 and MCF-7 cells were seeded in each well of a 96-well plate, incubated at 37°C, 5% CO<sub>2</sub> for 24 hours. The old culture medium was replaced by the medium containing extract at the IC<sub>50</sub> concentration or negative control DMSO. The test plate was incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. Each experiment was conducted in triplicate.

**Total RNA isolation.** Total RNA of the cells after treatment with P12 fraction or DMSO were isolated using TriSure (Bioline) according to the manufacturer's instructions. The extracted RNA was resuspended in 30µl of DEPC treated water and incubated for 10min at 60°C, and stored

at -80°C. The extracted RNA was quantified by optical absorbance (NanoDrop 1000. Thermo-Fisher Scientific, USA). The RNA extraction samples were then treated with DNase (Sigma Aldrich) to get rid of the genomic DNA.

**Synthesis of cDNA.** cDNA samples were synthesized by reverse transcription reactions using LunaScript RT SuperMix Kit 3010L (NEB, New England BioLabs) according to manufacturer's instructions.

**Relative quantification of mRNA levels by qPCR.** The real-time PCR (qPCR) method was performed with primer pairs for *survivin* (5'-TTGGCGGTCTAACTTAGCG-3' and 5'-CCTCGGCCAATCCGCTC-3') gene and the housekeeping gene GAPDH (5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'). The qPCR reaction was carried out using the Luna Universal qPCR Master Mix qPCR kit (NEB). The reaction compositions and thermal cycling conditions of the reaction were performed according to the manufacturer's instructions. The results of the qPCR reaction were processed using Prime Pro software. Thereby, the target gene expression (mRNA) of *C. quadrangulare* fraction-treated cells in comparison to DMSO was shown. The mRNA levels of the target gene will be calculated according to the formula (Livak & Schmittgen, 2001) based on the qPCR threshold cycle (Ct):

$$A = 2^{-\Delta\Delta Ct} \text{ (times)} \quad (2)$$

Where.

$$\Delta C_t (P12) = C_{t \text{ target gene}/P12} - C_{t \text{ GAPDH}/P12}.$$

$$\Delta C_t (\text{DMSO}) = C_{t \text{ target gene}/\text{DMSO}} - C_{t \text{ GAPDH}/\text{DMSO}}.$$

$$\Delta\Delta C_t = \Delta C_t (P12) - \Delta C_t (\text{DMSO}).$$

$C_t (P12)$ : threshold cycle with cells treated P12 fraction.

$C_t (\text{DMSO})$ : threshold cycle with cells treated DMSO.

$\Delta\Delta C_t$ : the difference of threshold cycle with cells treated P12 and DMSO.

### 3. Result and discussion

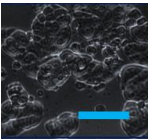
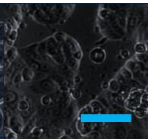
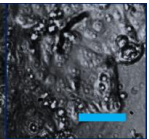

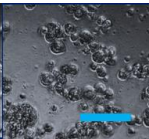
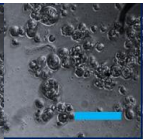
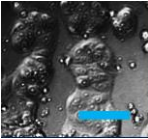
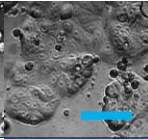
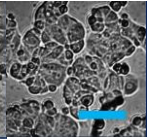
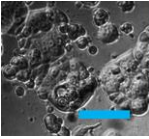
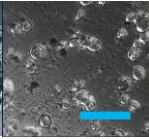
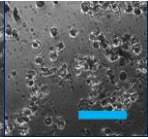
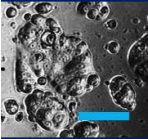
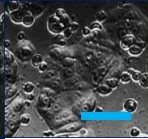
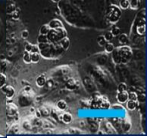
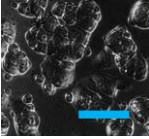
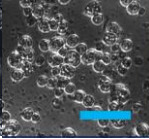
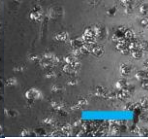
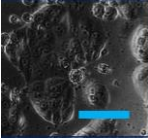
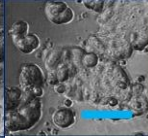
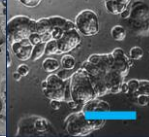
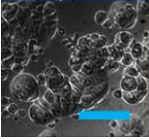
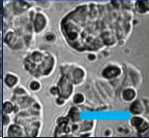
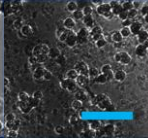

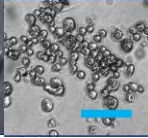
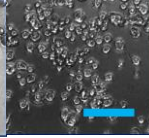
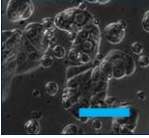
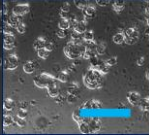
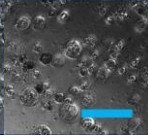
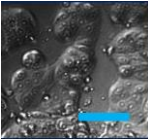
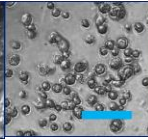
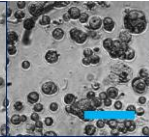
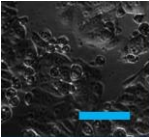
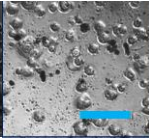
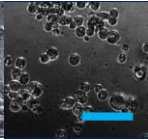
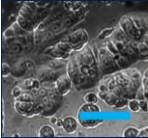
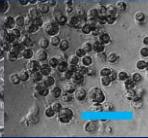
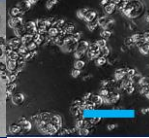
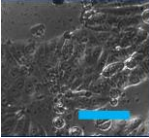
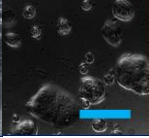
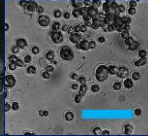
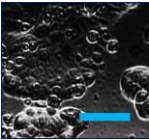
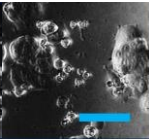
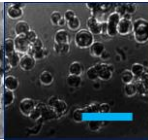
#### 3.1. Result

##### 3.1.1. Cytotoxicity of P fractions against MCF-7 cells

After 72 hours of culture with P1, P2, P3, and P4 fractions at a concentration of 50µg/ml, MCF-7 cells remained in the adhesion state. However, cells shrunk and turned into an unattached state after 48 hours of P5, P6, P7, P8, P9, P10, P12, and P13 fractions treatment. Meanwhile, cells were treated with P11, P14, and PAX1 only expressed the non-adhesion morphology after 72 hours of treatment. Therefore, P5, P6, P7, P8, P9, P10, P12, and P13 fractions had a better effect on MCF-7 cells in comparison to the others (Table 1).

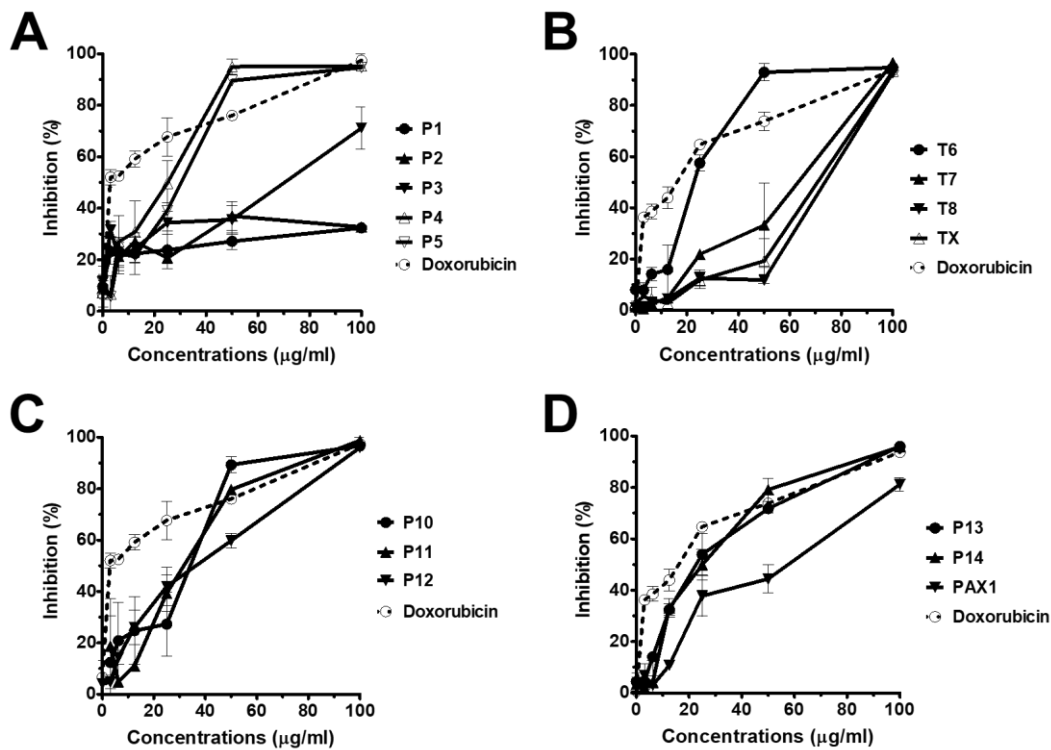
**Table 1**

Changes in MCF-7 morphology after treating P1-P14, PAX1 fractions at a concentration of 50µg/ml at 24, 48, and 72 hours of treatment

Fractions	Time (hours)			Fractions	Time (hours)		
	24	48	72		24	48	72
P1				P8			
P2				P9			
P3				P10			
P4				P11			
P5				P12			
P6				P13			
P7				P14			
				PAX1			

Source: Data analysis result of the research

According to Figure 1, P fractions were capable of causing toxic effects against MCF-7 cells. However, it was divided into two groups based on cytotoxicity potential. Weak cytotoxicity group containing P1, P2, and P3 fractions that almost did not affect MCF-7 cell growth. And strong cytotoxicity group consists of the other fractions that strongly affected MCF-7 cells growth. Among the strong cytotoxicity group, the percentage of dead cells increased rapidly at the concentration range from 12.5µg/ml for P6, P8, and P12. Thus, P6, P8, and P12 had a better effect on MCF-7 cells than the other fractions.



**Figure 1.** Cytotoxicity against MCF-7 of the fractions (A) P1- P5, (B) P6- P9, (C) P10- P12, (D) P13, P14, and PAX1, and positive control - Doxorubicin. The cell death rates are expressed as the mean of triplicate and Standard Deviation (SD)

Table 2 showed that P1 and P2 fractions did not affect cells proliferation. P8, P12, P13, and P14 were the fractions with a strong effect on cells proliferation. Among them, the P12 fraction from *C. quadrangulare* had the best cytotoxicity on MCF-7 with  $IC_{50}$  of  $20.9 \pm 0.8 \mu\text{g/ml}$  compared to other fractions. Thus, the P12 fraction was selected for the next experiment.

**Table 2**

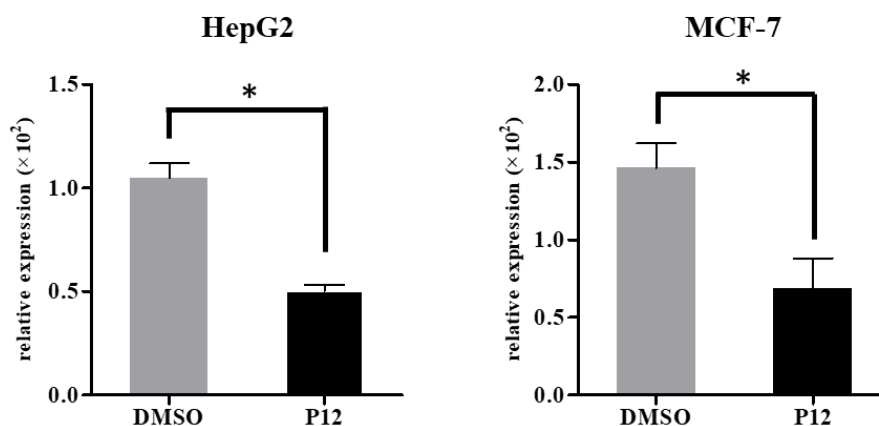
$IC_{50}$  of P1-P14 and PAX1 fractions of *C. quadrangulare* on MCF-7 cells

No.	Fractions	$IC_{50}$ ( $\mu\text{g/ml}$ )	No.	Fractions	$IC_{50}$ ( $\mu\text{g/ml}$ )
1	P1	>100	9	P9	$31.3 \pm 4.9$
2	P2	>100	10	P10	$33.67 \pm 2.7$
3	P3	$74.6 \pm 8.1$	11	P11	$31.3 \pm 3.3$
4	P4	$39.3 \pm 1.4$	12	P12	$20.9 \pm 0.8$
5	P5	$54.1 \pm 3.8$	13	P13	$23.8 \pm 5.0$
6	P6	$55.9 \pm 3.9$	14	P14	$25.2 \pm 4.0$
7	P7	$33.1 \pm 2.0$	15	PAX1	$56.8 \pm 5.4$
8	P8	$24.2 \pm 3.9$	16	DOX	$13.9 \pm 2.2$

Source: Data analysis result of the research

### 3.1.2. Effect of P12 fraction on survivin mRNA levels of liver and breast cancer cells

The results presented that mRNA levels of anti-apoptotic gene *survivin* decreased significantly in both P12-treated HepG2 and MCF-7 cells in comparison to DMSO-treated cells (Negative Control, NC). In particular, mRNA levels of *survivin* gene decreased in a factor of  $2.1 \pm 0.23$  and  $2.38 \pm 0.79$  of P12-treated HepG2 and MCF-7 cells, respectively, compared to DMSO-treated cells. Thus, with the premise that the decreased expression levels of *survivin* (an inhibitor of apoptosis gene) in P12-treated liver cancer and breast cancer cells, we predicted that the P12 fraction would be cytotoxic to HepG2 and MCF-7 cells by the mechanisms of apoptosis.



**Figure 3.** Relative expression of *survivin* gene in P12-treated cancer cells in comparison to DMSO-treated cancer cells. \*,  $p < 0.05$

### 3.2. Discussion

Regarding anticancer activity of the extracts and compounds from *C. quadrangulare*, the studies performed on human lung cancer (Chittasupho & Athikomkulchai, 2018), hepatocarcinoma, and colon cancer (Nantachit et al., 2015), murine colon carcinoma cells (Adnyana, Tezuka, Banskota, Tran, & Kadota, 2001) showed good effects. According to Roy et al. (2014), isolated compounds *C. quadrangulare* from containing up to 75 compounds of the Triterpenoids group, 15 compounds of Flavonoids groups, and some miscellaneous (Roy et al., 2014). In addition, Banskota, Tezuka, Adnyana, et al. (2000) reported the 19 compounds isolated from *C. quadrangulare* that had a cytotoxic effect on murine colon 26-L5 carcinoma cells in which 15 compounds belong to Triterpenoids and four compounds of Flavonoids (Banskota, Tezuka, Adnyana, et al., 2000; Banskota et al., 1998; Banskota, Tezuka, Tran, et al., 2000a; Banskota, Tezuka, Tran, et al., 2000b). A study showed that one triterpene and three flavonols enhanced DR5 expression, and one flavonol showed TRAIL-resistance abrogating activity that triggered apoptosis of cancer cells (Toume et al., 2011). This study provided more proof of the cytotoxicity potential of *C. quadrangulare* Kurz against cancer cells.

According to Boykin et al. (2011), a triterpenoid induced apoptosis in cancer cells by regulating PARP expression levels through inhibition of *survivin* levels of JAK2/STAT3 downstream pathway. On the other hand, a study was performed to report two bioactive triterpenoids were isolated from the P12 fraction (Nguyen et al., 2021). This study is, to the best of our knowledge, the first identified down-regulation of *survivin* expression levels under the influence of *C. quadrangulare* extract - P12. So, we can expect P12 fraction inhibited *survivin* expression levels because of partial triterpenoids.



The extract can induce apoptosis of cancer cells in many ways related to survivin expression levels. Basically, an extract can directly inhibit survivin, leading to up-regulating the expression levels of caspase-3, -8, -9, and Bid proteins (Conway et al., 2002; Chandele et al., 2004). A study by Dhaheri et al. (Al Dhaheri et al., 2013) proposed that breast cancer cells apoptosis can be triggered by *Origanum majorana* extract via down-regulation of survivin and mutant p53. Although it is not possible to probe this directly by the current study, our results may indicate the possibility of such a mechanism because of a high frequency of this downstream signaling pathway. Furthermore, in the upstream signaling pathway of apoptosis, the extract can act like triterpenoids suppressing JAK2/STAT3 expression levels, causing down-regulation of survivin, resulting in apoptosis of cancer cells (Boykin et al., 2011). Herein, the P12 fraction was exhibited the apoptosis triggering mechanism by down-regulating survivin expression levels, and we expect further signaling pathways that P12 provoke apoptosis in liver and breast cancer cells.

#### 4. Conclusions & recommendations

In this study, we evaluated the cytotoxicity of P fractions from *Combretum quadrangulare* Kurz. In addition to a previous study (Nguyen et al., 2021), the P12 fraction was identified as a potential cytotoxic fraction against liver and breast cancer cells. Furthermore, the P12 fraction induced apoptosis in cancer cells by downregulating the expression levels of the *survivin* gene. Thus, we expect that figure out the upstream signaling pathway that P12 has an effect on liver and breast cancer cells.

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