# Ethyl Acetate Fraction of *Andrographis paniculata* Nees Increases Cytotoxic Effect of Vincristine on Human Cancer Cell Lines

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**Abstract**

Objective: The objective of the present investigation was to examine whether ethyl acetate fraction from *Andrographis paniculata* Nees (EAA) synergizes the therapeutic potential of vincristine (VCR) against different human cancer cell lines (T47D, HeLa, and WiDr).

Materials and Methods: MTT assay was used to measure the growth inhibitory effect of the combination. Synergistic efficacy was subjected to median effect analysis with nonexclusive model as previously described by Chou and Talalay.

Result: $IC_{50}$ of EAA were 18.486 µg/ml in HeLa cells line, 13.467 µg/ml in Widr cells line and 21.618 µg/ml in T47D cells line. EAA and VCR combined as a cocktail, synergistically inhibited the growth of cancer cells in vitro, with Combination Index value (CI) ranging from 0.975 to 0.224 in T47D cells line, 0.447 in HeLa cells line and 0.887 to 0.326 in WiDr cells lines.

Conclusion: EAA and VCR, combined as a cocktail, showed synergism in inhibiting the growth of human breast cancer cells (T47D) in vitro.

**Key words**: *Andrographis paniculata* Nees, Vincristine, Ethyl Acetate Fraction, Human Cancer Cell Lines

## INTRODUCTION

Cancer is a disease characterized by a shift in the control mechanisms that govern cell survival, proliferation, and differentiation (Trevor *et al*., 2007). Chemotherapy has undergone a gradual transition from mono-substance therapy toward multidrug therapy, and drug cocktails strategy has become widely adopted. Properly formulated drug combinations are believed to enhance synergism and the interactions of chemical components within the combination may improve therapeutic efficacy over single drugs (Sandler *et al*., 2006) and in many cases plant extracts are thought to be therapeutically superior to their single isolated constituents (Mijatovic *et al*., 2011; Wagner *et al*., 2005). Therefore, herbal medicines are increasingly combined with chemical medicines in anticancer drug cocktails, especially in countries where herbal medicines are well accepted (Lei *et al*., 2008; Hsiao *et al*., 2010). Some studies have suggested that for cancer treatment, drug cocktails combining herbal and chemical medicines may exhibit enhanced efficacies with diminished side effects and complications (Qi *et al*., 2010; Hermawan et al., 2012)

*Andrographis paniculata* Nees (Acanthaceae) is a traditional medicinal herb, grown as shrub in the moist soil, shady areas of India, China, Indonesia and throughout Southeast Asia. It has been used as immunostimulant (Puri *et al*., 1993), for myocardial ischemic (Guo *et al*., 2005) pharyngotonsillitis (Thamlikitkul *et al*., 1991) respiratory tract infections (Coon *et al*., 2004) and common cold (Melchior *et al*., 1996). It also possesses antimicrobial effect (Prajjal *et al*., 2003) anti-inflammatory (Shen *et al*., 2002), hypotensive effect (Zhang *et al*., 1996) antihyperglycemic (Borhanuddin *et al*., 1994; Yu *et al*., 2003) atherosclerotic (Wang *et al*., 1994) antimalarial activity (Dua *et al*., 2000), anti-HIV (Calabrese *et al*., 2000), antiplatelet aggregation (Amroyan et al., 1999), hepatic lipid peroxidation protective (Choudhury *et al*., 1984), hepatoprotective (Handa *et al*., 1990), choleretic effect (Shukla *et al*., 1992) and anticancer effects (Chang *et al*., 1987; Rajagopal *et al*., 2003; Kumar *et al*., 2004). One of the major constituents of *A. paniculata* Nees is diterpene lactone such as andrographolide, which has anticancer activity *in vitro* in many tumor cell lines including leukemia, myeloma, HeLa, colon (HT-29), human peripheral blood lymphocytes (HPBLs), and human breast cancer MCF-7 (Satyanarayana *et al*., 2004). It was found that andrographolide possessed inhibitory effect of DNA Topoisomerase II (Sukardiman *et al*., 2005). It reported that andrographolide inhibition of cell cycle from human breast cancer cell MCF-7 by induction of cell-cycle inhibitory protein p27 also decreased expression of cyclin-dependent kinase. Andrographolide isolated from *Andrographis paniculata* Nees induced apoptosis in T47D human breast cancer cell line in a time and concentration-dependent manner by increase expression of p53 bax, caspase-3 and decrease expression of bcl-2 (Sukardiman *et al*., 2007).

Vincristine is an alkaloid isolated from the leaves, bark, or stem of the Madagascar periwinkle (*Catharanthus roseus*) (Tyagi *et al*., 2010). It has also been used in combination with other antineoplastic drugs in Hodgkin's disease, sarcomas of specialized structure, breast cancer, cancer of the uterine cervix, malignant melanoma, colorectal cancer, and Wilm's tumor (Lassen *et al*., 1996).

The aim of this paper is to evaluate the efficacy of the ethyl acetate fraction of *Andrographis paniculata* Nees (EAA) as a source of useful anticancer agents and the co-efficacy at the cellular level of a cocktail combining EAA and VCR.

## MATERIALS AND METHODS

**Plant Material.** *Andrographis paniculata* Nees herb were obtained from Mojokerto, East Java area, which was then determined by the Department of Pharmacognocy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Surabaya Indonesia.

**Preparation of Ethyl Acetic Fraction ( EFA).** The extract was prepared by macerating dried aeral part powder in 95% ethanol. The macerate was then concentrated under vacuum rotary evaporator, and ethanolic crude extract was separated using ethyl acetate and water. Fraction of ethyl acetate was then concentrated under vacuum rotary evaporator. The concentrated fraction was then prepared in DMSO (Sigma) for treatment. The final DMSO concentration was set not higher than 0.1 %

**Cell Lines.** Widr, T47D and HeLa cells were cultured in RPMI Medium containing Fetal Bovine Serum (FBS) 10% (v/v) (FBS qualified, Gibco, Invitrogen TM USA) and penicillin-streptomycin 1 % (v/v) (Gibco, In vitro gen Corporation, Grand Island, NY, 14072, USA). These cell lines were kindly provided by the Department of Parasitology, Faculty of Medicine, Gadjahmada University, Yogyakarta, Indonesia.

**Drugs.** Vincristine sulphate i.v (vial 2 mg/2 ml) purchased from P.T. Kalbe Farma (Cikarang, Indonesia) was diluted directly in culture medium.

**Cytotoxic Assay-MTT Method.** T47D, HeLa, and WiDr cells ($10^4$cells/well) were transferred into 96-well plate and incubated for 24 hours (70-80% confluent). Cells were treated by EAA, VCR, and their combination, then incubated for 24 hour. At the end of the incubation, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] 0.5 mg/ml were added to each well followed by 4 hours incubation in 37°C chamber. Viable cells react with MTT to form purple formazan crystals. After 4 hours, stopper sodium dodesil sulphate (SDS) 10% in 0,1 N HCl solution were added to dissolve the formazan crystals. Following overnight incubation (with protection from light exposure), the cells were shaken for 10 minutes before being read by ELISA reader at λ 595 nm. The obtainedabsorbance of each well converted to percentage of viable cells:

$$\% \text{ viable cells} = \frac{\text{Treated cells abs} - \text{Mediumcontrol abs}}{\text{Cell control abs} - \text{Medium control abs}} \times 100\%$$

**Combination index (CI) for determining synergism additivity or antagonism.** The combined effects of EAA and VCR were subjected to median effect analysis with the mutually nonexclusive model as previously described [1]. The combination index (CI) for determining synergism and antagonism between the substances was calculated using SPSS 20.0; SPSS Inc.). CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism respectively. The results by ATP assay were analyzed for CI determination.

## RESULTS AND DISSCUSSION

**Component identification of EAA Fraction by TLC fingerprint.** Chromatographic fingerprinting is a powerful technology for authentication of natural products. The application of chromatographic fingerprinting in component identification of natural products continues to expand. TLC – Densitometry fingerprinting of EAA fraction for quality control is shown in Figure 1. The 3 main compounds of EAA fraction found in this study , with major compound is andrographolide.

**Cytotoxic Assay.** To explore the effects of EAA fraction on human cancer cell lines and normal cells in vitro, the cytotoxicity of EAA fraction at 10 – 100 µg/ml for 24 h was assessed by MTT assays in a panel of human cancer cell lines namely HeLa, Widr and T47D. Figure 2 shows that growth was strongly inhibited in all cancer cells with $IC_{50}$ of EAA are 21.379 µg/ml in T47D cells, 18.109 µg/ml in HeLa cells and 14.520 µg/ml in WiDr cells. Therefore, EAA showed strong and broad-spectrum anticancer activity. But $IC_{50}$ of VCR in different human cancer cell lines are 27.911 µg/ml in T47D cells , 2.115 µg/ml in HeLa cells and 28.215 µg/ml in WiDr cells. A previous study of VCR cytotoxicity on T47D and Widr cells showed a weak cytotoxicity on HeLa and Widr cells with $IC_{50}$ 27.911 µg/ml and 28.215 µg/ml (Fig 3).

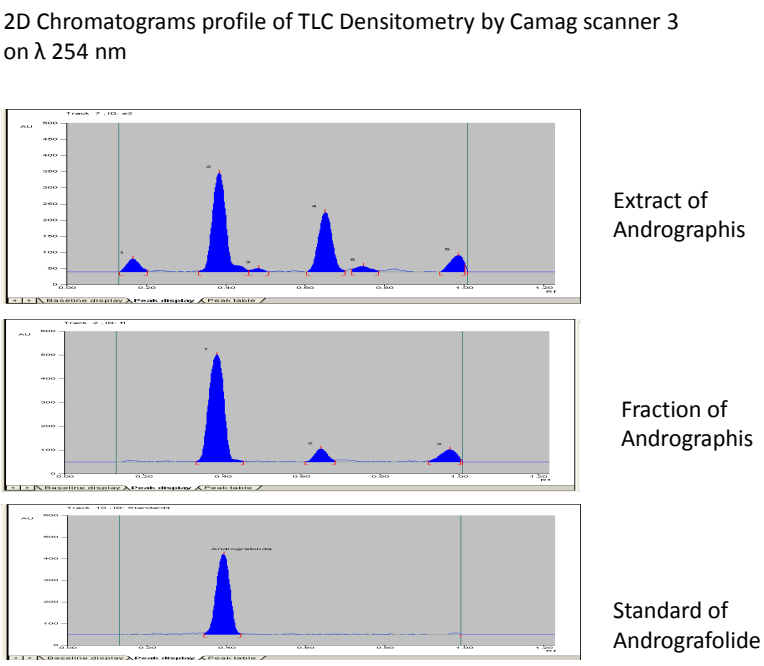


**Figure 1**. TLC-Densitometry fingerprint from EAA fraction with eluen choloform –methanol (9:1) by λ=254nm

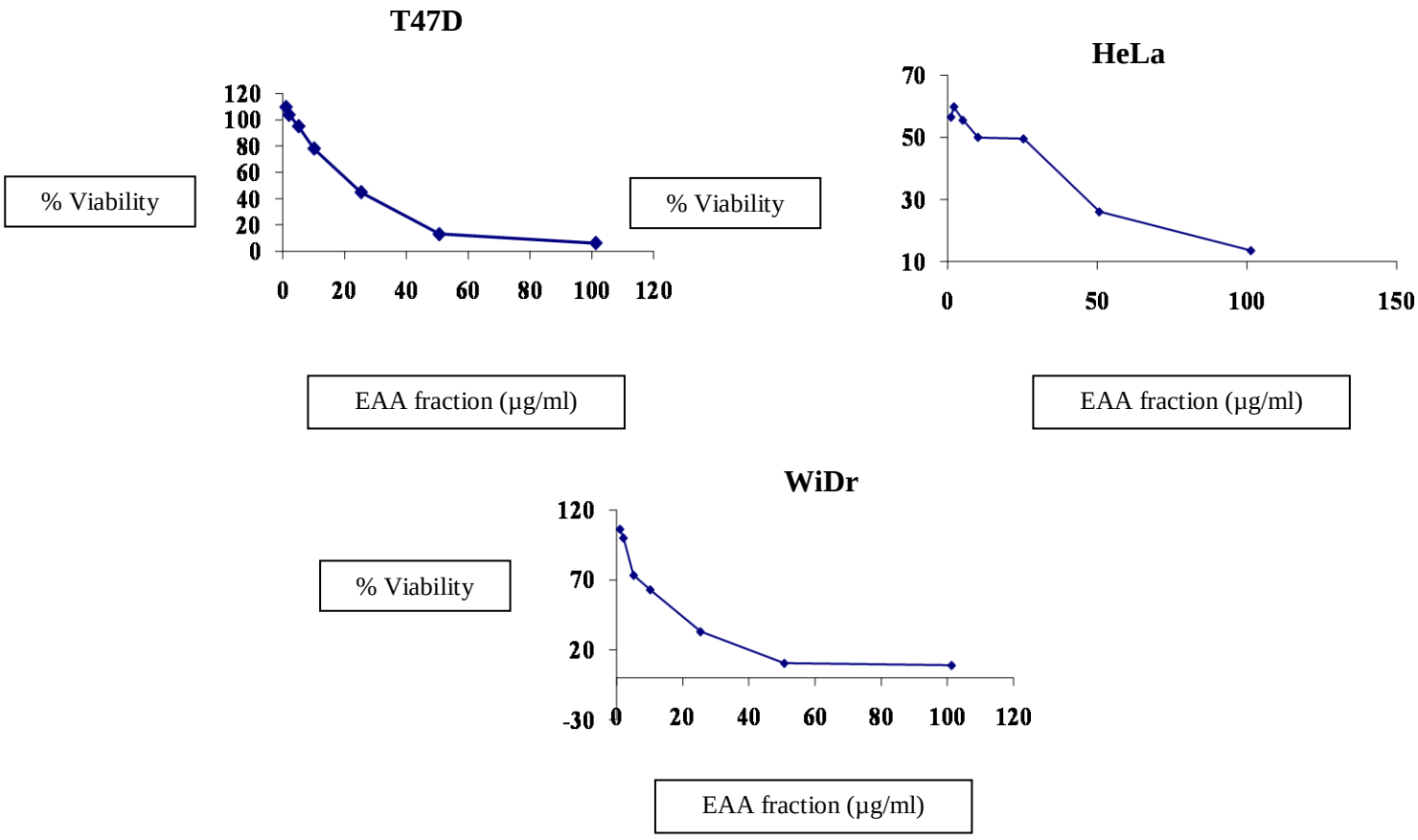


**Figure 2**. Differences in EAA fraction cytotoxicity in 3 human cancer cell lines. The cell growth inhibition was quantified by the MTT assay. Cells were treated with 10 – 100 µg/ml EAA fraction for 24 h. The data shown are from 3 independent experiments.

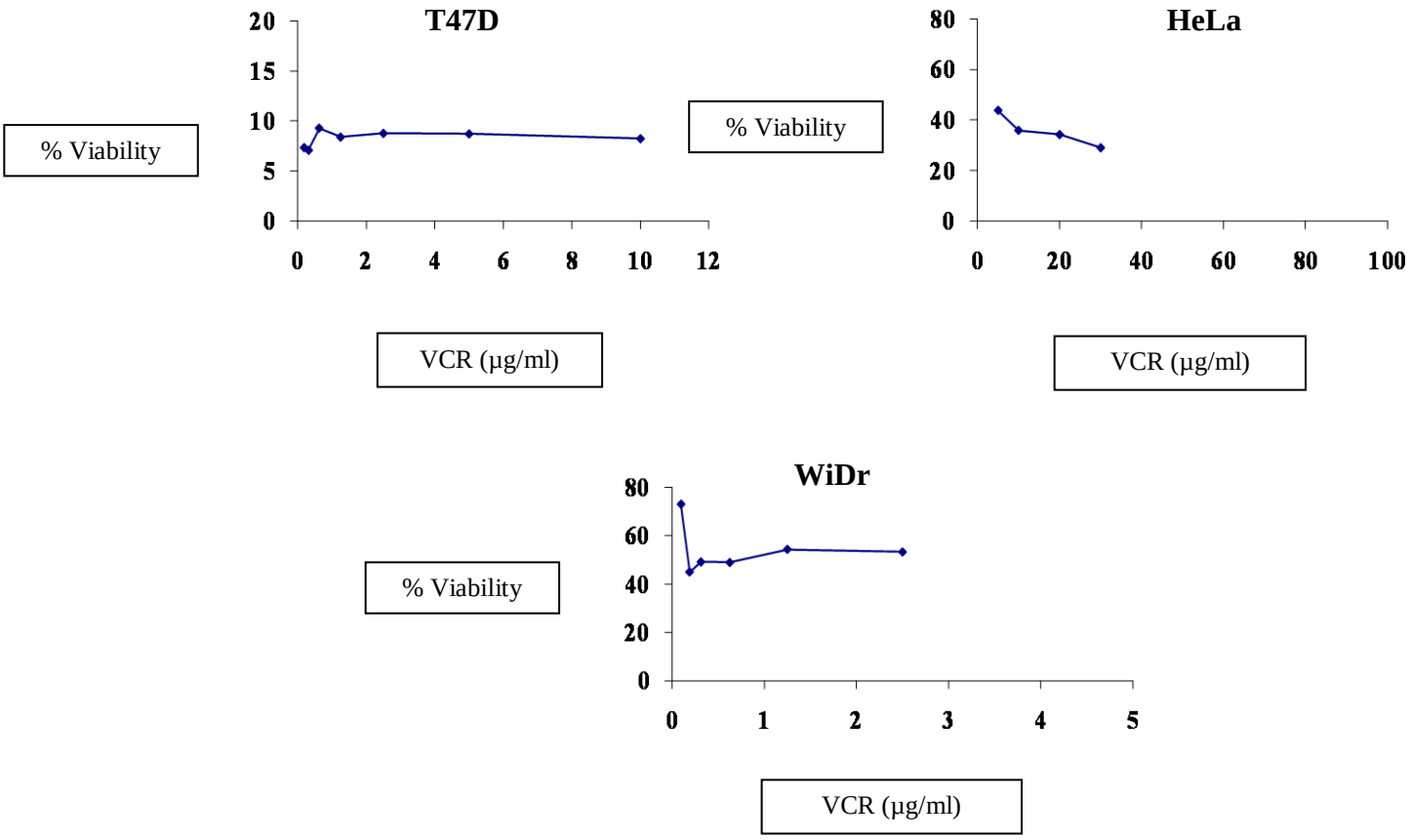


**Figure 3**. Differences in VCR cytotoxicity In 3 human cancer cell lines. The cell growth inhibition was quantified by the MTT assay. Cells were treated with 0.09375-30 µg/ml VCR for 24 h. The data shown are from 3 independent experiments.

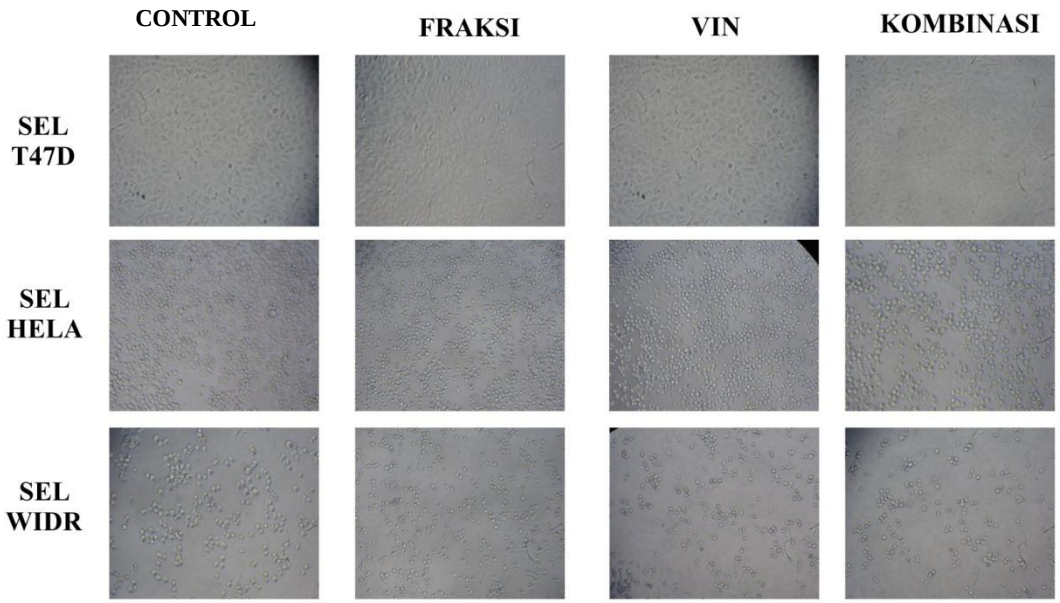


**Fig 4**. The effect of EAA fraction and VCR alone and combination of EAA fraction and VCR to the morphology of T47D, HeLa, and Widr . Cell morphology was examined by using inverted microcope with magnification 400x.

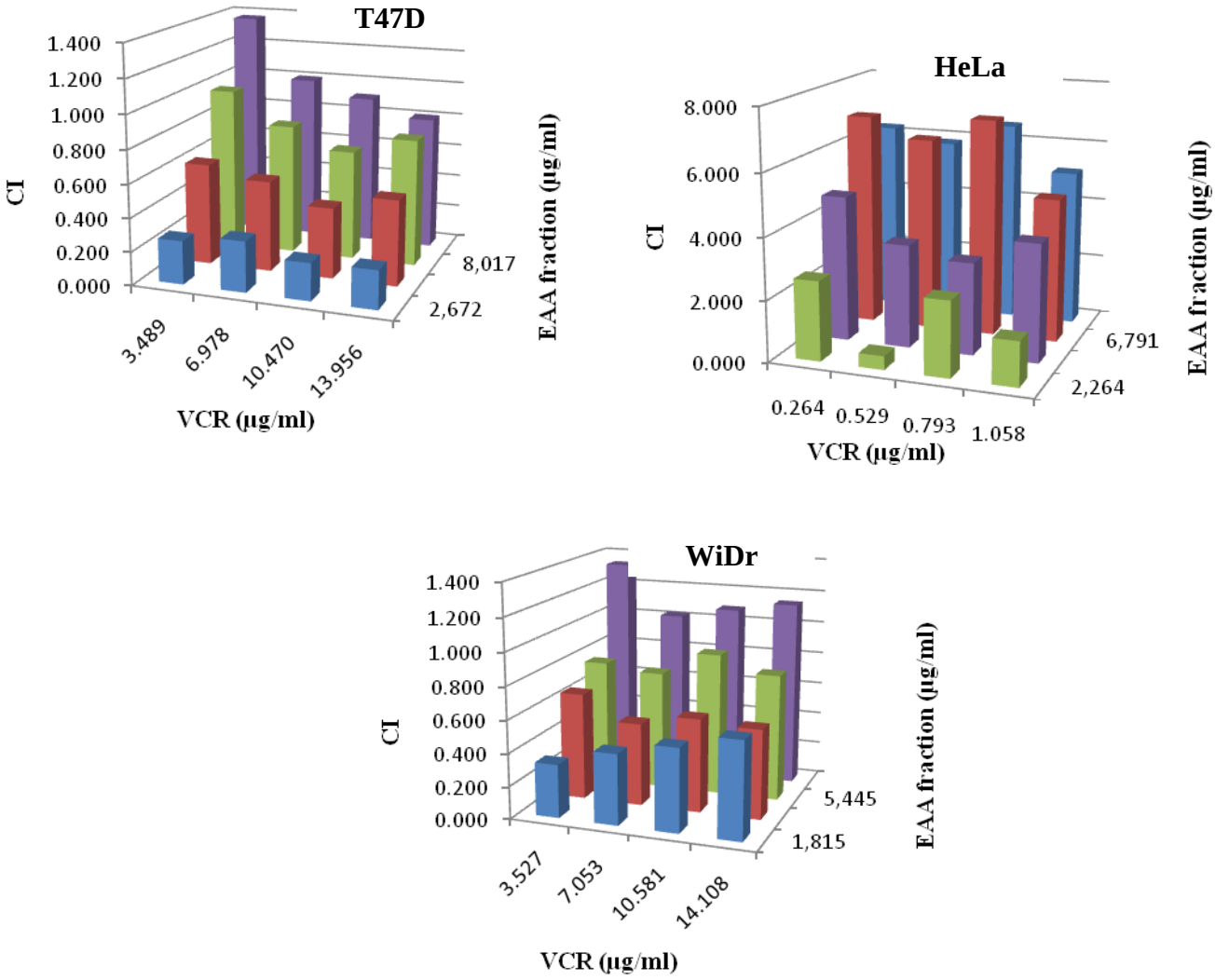


**Figure 5.** Synergistic antitumor effect of the combination of EAA fraction and VCR in Human Cancer Cell Lines

**Combination of VCR and EAA fraction produced synergistic effects on human cancer cells.** In order to investigate the anticancer activity of a cocktail containing EAA fraction and VCR, cytotoxic studies were performed in human cancer cell lines (T47D, HeLa, and WiDr). As shown in Fig 4. the viability levels of all cell lines decreased.

To determine whether the combined effects of the EAA fraction and VCR were synergistic, the CI value was calculated where CI < 1, = 1, and > 1 represent synergism, additive effect, and antagonism, respectively. EAA and VCR, combined as a cocktail, synergistically inhibited the growth of cancer cells in vitro, with Combination Index value (CI) ranging from 0.975 to 0.224 in T47D cells line, 0.447 in HeLa cells line and 0.887 to 0.326 in WiDr cells lines EAA and VCR, combined as a cocktail, strong synergistically inhibited the growth of human breast cancer cells (T47D) in vitro (Fig 5).

The present study explored the effect of EAA fraction alone and in combination with VCR on cytotoxicity of T47D, HeLa and WiDr cells. Single treatment of EAA fraction showed potent cytotoxic effect, but VCR did not show potent cytotoxic to T47D and WiDr cells, while combination with VCR increased cytotoxic effect of VCR. These results are interesting to be evaluated. Combination of EAA and VCR probably increased EAA and VCR intracellular concentrations. Previous study reported chemotherapy drug induced cell membrane peroxidation leads to membrane leakage and increased transport EAA into cells (Minotti et al., 2004). The present of TLC–Densitometry fingerprinting of EAA fraction that shown 3 main compounds of EAA fraction found in this study, with major compound active is andrographolide, with have strong anticancer effect in T47D, HeLa and WiDr.

This result showed that combination of EAA fraction and VCR strongly synergistic in inhibited the growth of cancer cells. VCR perturb the kinetochore microtubule attachment. This activates a checkpoint pathway that ensure proper attachment of

chromosomes to the mitotic spindle (Amon, 1999; Rudner, 1996; Burke DJ, 2000). When microtubules fail to attach to one or more kinetochores as a result of drug treatment, the checkpoint components continue to generate signals that inhibit the metaphase/anaphase transition that delays cell cycle progression and induces programmed cell death (Sorger et al.,1997) (b) inhibition of cell growth and induction of apoptosis were synergistic or additive but not antagonistic; (c) Bax played a key role in andrographolide /VCR related apoptosis; (d) mitochondrial membrane potential significantly disappeared after combination treatment; (e) p53 may also play a role in the apoptosis induced by the combination.

The present study, showed the potency of *Andrographis paniculata* Nees to be developed as a co-chemotherapeutic agent for VCR. The use of VCR together with EAA is expected to increase the activity and reduce the side effect of VCR. However, the molecular mechanism of cytotoxic effect by this combination need to explored in detail.

## CONCLUSION

The research showed that combination of EAA and VCR increased the effect of VCR against various human cancer cell line (T47D, HeLa, and WiDr).Based on this result, EAA is potential to be developed as a co-chemotherapeutic agent for VCR in cervical, colon and breast cancer therapy.

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