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The cytotoxic and apoptotic effects of wild and polyploidy genotype of *Artemisia cina* extracts on the WiDr colon and HTB-183 lung cancer cell lines

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Abstract. *Kristiani EBE, Kasmiyati S, Herawati MM. 2021. The cytotoxic and apoptotic effects of wild and polyploidy genotype of* Artemisia cina *extracts on the WiDr colon and HTB-183 lung cancer cell lines. Biodiversitas 22: 2844-2852. Artemisia* belongs to the Asteraceae family, usually used for traditional treatments of various diseases in China. *Artemisia cina* Berg ex Poljakov, which lots found in Indonesia, has not been studied much like a cancer drug. The research aims were (i) to compare the bioactive content of hexane and ethyl acetate extract of wild (TWN and KJT) and polyploidy type (J and M) of *A. cina* and its cytotoxicity on WiDr and HTB-183 cancer cells, (ii) to evaluate the cytotoxicity mechanism of the most substantial extract of it. All of the extracts were prepared by maceration methods using hexane and ethyl acetate separately. The research method used was quantitative with experimental design. The determination of quercetin, kaempferol, and artemisinin used HPLC. The cytotoxicity of the extract determined using the MTT method. The assay of the specific protein related to apoptosis using ICC assay. The content of three bioactive compounds on ethyl acetate extracts higher than hexane extract, in which the extract of J and KJT genotypes had higher levels than M and TWN genotypes. The IC_{50} value (µg/mL) of extracts against cancer cell lines tested was about 400-700 hexane extracts and 200-500 ethyl acetate extracts. The BCl-2 expressions of both cancer cell lines decreased by treating TWN-EA and M-EA, while the P53 expressions increased. The TWN-EA induced apoptosis of HTB-183 cell lines through caspase-8 and caspase-9 pathways, but M-EA induced caspase-9 pathway only. The TWN and M genotypes were potential to use as anticancer agents on colon and lung cancer.

Keywords: *Artemisia cina*, apoptosis, Bcl-2, caspase, cancer, cytotoxicity, p53

Abbreviations: B2P2TOOT: Balai Penelitian dan Pengembangan Tanaman Obat dan Obat Terstandar; MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; ICC: Immunocytochemistry, HPLC: High-Pressure Liquid Chromatography, TWN-EA: ethyl acetate extract of TWN; M-EA: ethyl acetate extract of M

INTRODUCTION

According to World Health Organization (WHO), cancer is one of the leading causes of death worldwide, with around 9.6 million deaths in 2018. From that, lung cancer is the first (2.09 million cases), and colon cancer is the third (1.80 million cases) cause (WHO 2018). Colorectal cancer (CRC) becomes the third cause of death by cancer cases in the United States of Amerika. National Center for Health Statistics reported that in 2020, it is about 147,950 CRC cases, and 40% died (Siegel et al. 2020). About lung cancer, this cancer as the most leading of death caused by cancer in China in recent years (Cheng et al. 2020).

Cancer treatments that are primarily carried out include surgery, radiation and chemotherapy, which are very high so that it is a burden to the patient concerned or the country and still causes side effects (Abdullah et al. 2014). It encourages exploring new drugs by using the natural material approach that has traditionally been used as medicine (ethnobotanical bioprospecting approach) (Kashani et al. 2012). Many countries had an endemic plant usually used as medicinal plants, such as *Albertisia papuana* Becc. and others in Indonesia (Kristiani et al. 2016; Sholikhah 2016). *Heracleum platytaenium* and others in form Turkey (Tuzlaci and Senkardes 2011; Akillilar et al. 2018), in China, included *A. capillaries* Thunb, *A. centifolia* Buch-Ham. Ex Roxb, *A. japonica* Thunb, etc. (Gao et al. 2019). The pharmacological ability of herbal medicines is influenced by their bioactive compounds, which are usually secondary metabolites (Kashani et al. 2012). The type of bioactive compound carried in an extract is closely related to the solvent used to extract the sample (Dai and Mumper 2010). Most of the techniques are based on the kind of solvent, the time of mixing, and the temperature of the process (Naboulsi et al. 2018; Zhang et al. 2018). Solvent determination is very crucial in the extraction process. Several properties such as selectivity, solubility, cost and safety are considered when selecting solvents. The likes to dissolve like principle describes the polarity of the solvent will affect the polarity of the extracted compound (Zhang et al. 2018). A study of *Severinia buxifolia* (Rutaceae) showed that the influence of the used solvent on the many essential roles included the

yield of extraction, the content of bioactive components, and biological activities. The kind of solvent used was distilled water (H_2O), MeOH, EtOH, $CHCl_3$, CH_2Cl_2, and Me_2CO (Zhang et al. 2018). Ngo et al. (2017) used seven solvents with different polarity indexes are 10.2, 5.1, 4.3, and 5.1, respectively, to extract the bioactive compounds from *Salacia chinensis* L. root. It shown that acetone (50% v/v) was found to be the optimal extraction solvent for extractable solids 50% acetone is recommended for the extraction of phenolic compounds, their secondary metabolites, saponins, and antioxidant capacity from the root of *S. chinensis* for further isolation and utilization.

Based on their structure, they are divide into three groups that are terpenes and compounds contained N/S and phenolics compounds. (Pagare et al. 2015). Flavanoids are a member of the phenolic compound group with a wide range of function in the plant. The type of secondary metabolite compounds can be extracted using extraction techniques. Many secondary metabolites were shown to include polyphenolic compounds, including flavonoids, tannins, curcumin, resveratrol and gallocatechin, and brassinosteroids (Greenwell and Rahman 2015). Shree et al. (2019) represented that terpenoids, flavonoids, phenols and alkaloids were compounds that could be anticancer agents. Wang et al. (2012) stated that there are various anticancer compounds, including kaempferol and triterpenoids.

In the last decade, various research results have shown that certain compounds in plants exhibit cytotoxic activity against cancer cells so that they can be used as a source to obtain new cancer drugs (Arullappan et al. 2013). Artemisia belongs to the Asteraceae family, which usually used as medicine. *Artemisia annua* has long used in China for traditional treatments of various diseases. Previous studies have shown that these bioactive compounds in Artemisia have antimicrobial, antimalarial, antioxidant, anthelmintic, and anticancer effects (Koul et al. 2017; Kasmiyati et al. 2020). Kursat et al. (2015) was identified that the quercetin and kaempferol content in five types of Artemisia was similar to *A. spicigera* and *A. splendens*. They were found that the two compound. Both compounds were not found in *A. haussknechtii* Boiss. The quercetin content in *A. armeniaca* Lam., *A. Incana* (L.) Druce, *A.tournefortiana* Reichb., and *A. scoparia* Waldst. & Kit was about 0.001 – 0.065 %, while the kaempferol content was about 0.003 – 0.02 % in *A. Armeniaca* Lam, *A. tournefortiana* Reich, and *A. scoparia* Waldst. & Kit only. Identification of *A. absinthium* leaf by Lee et al. (2003) showed that the content of quercetin and kaemferol were low too, that was 0.0031 % and 0.0013 %, respectively. The artemisinin concentration on Artemisia was influenced by altitude and soil (Mukazayire et al. 2009). They were found that artemisinin content on *A. annua* was grown at higher 2000 m, 1800 m, and 1650 m were 1.11 %, 1.17 % and 0.46 %, respectively. The content of artemisinin varied in every part of the plant to flowers, leaves, stems, and roots. Those reports informed the possibility of *Artemisia* as an anticancer agent.

The phytopharmaceutical compounds of *Artemisia* are very potential, but are small. Tetraploid induction of this plant increased the secondary metabolites content of plants (Carusoa et al. 2013). The artemisinin content of this species very little is about 0,0075-0,66%. Tetraploid induction of this plant increased the artemisinin content three times than the diploid plant (Herawati et al. 2003). The increased levels of artemisinin may allow changes in the level of other secondary metabolites. The increase of bioactive compounds will affect its cytotoxic capability, and thus, this research will measure the anticancer bioactive compounds of the wild and polyploidy type of *Artemisia cina* Berg ex Polyakov. The previous research has shown that out of the four cancer cells observed, the toxicity level of this plant against colon cancer cell WiDr, and lung cancer cell HTB-183, is more substantial than it is against breast cancer cells T47D and cervix cancer cell HeLa. Because the ability to modulate a cell's life and death is the most sought-after potential in anticancer studies, cell cycle and apoptosis mainly focus on those studies (Elmore 2007).

Based on that, the research aims were (i) to compare the cytotoxicity and bioactive content of hexane and ethyl acetate extract of wild and polyploidy type of *A. cina* on WiDr and HTB-183 cancer cells and (ii) to evaluate the cytotoxicity mechanism of the most substantial extract of it.

MATERIALS AND METHODS

Materials

The method of research used was quantitative with experimental design. There were two kinds of genotypes of *Artemisia cina* used that was the wild type and polyploidy mutant. Two kinds of wild types genotypes were including TWN and KJT. TWN was a diploid genotype plant derived from the Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT) Tawangmangu, Centre of Java, Indonesia, directly grown Saharan greenhouse. KJT was a diploid genotype tissue culture with no induction. J and M were polyploid genotypes. J obtained by inducing *A. cina* to shoot culture with two mg/l of 2.4 D and three mg/l of IBA for 21 days, while M obtained by two mg/l of 2.4 D and 1.5 mg/l of IBA for 21 days (M). The *A. cina* plant collection (No. 001 until No. 004/2014/FPBUKSW/Koleksi) was preserved in the laboratory of the Faculty of Agricultural and Bussiness, Satya Wacana Christian University, Salatiga, Indonesia. The WiDr and HTB-183 cancer cell lines were obtained from Parasitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Preparation of extract

The whole part of the plant was air-dried, followed by counting using a blender (Philip HR1538). The extracts were prepared using hexane and ethyl acetate as the solvent, separately and accompanied by stir using the magnetic stirrer. The maceration process was repeated five times. Every process was carried out for about three hours, and the filtrate was filtered using filter paper. The combined filtrate was dried using a rotary evaporator

(Rotavapor R114 Buchi) under vacuum (Eyela A-1000S) at 40°C.

Determination of bioactive compounds using HPLC

Quercetin and kaempferol (Tokuşoğlu et al. 2003 with a slight modification)

The sample is converted into flavonoid glycosides form, and then the glycosides are hydrolyzed to form an aglycon flavonoid before being injected into the HPLC. Samples were added with methanol containing 1% HCl (1: 1 w / v) and sonicated (SonicatorKrisbow DSA50-GL2-2,5L) for 30 minutes. The mixture filtered; part of the filtrate is taken (as a glycoside flavonoid extract) hydrolyzed. The glycoside flavonoid extract was saturated to a particular volume and added with 1.2 M HCl as much as 20% of the volume of the extract's fulfillment, then refluxed at 90ºC for 2 hours. After the extract cooled, sonication was continued for 3 minutes and then filtered using a 0.45 µm filter membrane. The filtrate obtained is an aglycon flavonoid and is ready to be injected into HPLC (*Knauer Germany Series Smartline)*. The modifications of operational conditions of the HPLC include use of Chromosorb RP C18 (150x5 mm id) Knauer as a column, 0.1% H_3PO_4 acetonitrile (60:40) as mobile phase with flow rate was 1 mL/mnt, an ambient temperature, and UV 370 nm as a detector. The volume of injection was 20 µL: pure quercetin, and pure kaempferol used as standard compounds.

Artemisinin (Numonov et al. 2019 with modification)

The 100 mg powdered sample was added with 100 mg granular quartz and 2 mL toluene and then crushed using mortar. The filtrate was filtered and stored in a container. The 500 µL filtrate was evaporated to dryness and then re-dissolved using 200 µL methanol and 800 µL NaOH 0.2 %w/v. The solution was agitated using a vortex mixer (Scilogex Type MX-S)) and then heated using a water bath (Memert) in 50ºC for 30 minutes and cooled again. After this, the solution was homogenized with 200 µL methanol and 800 µL acetic acid and then filtered using a 0.45 µm membrane filter. The filtrate in the form of artemisinin was ready to be injected into the HPLC. The modification of HPLC conditions was included the Chromosorb column RP C18 (150x5 mm id), Knauer, the mobile phase was buffer Phosphate 0,01 M pH7: methanol (55:45), the flow rate was 0.5 mL/min, the volume of injection was 20 µL, ambient temperature, and using UV 260 nm detector. The pire artemisinin was used as standard compounds.

Cytotoxic assay

The cytotoxicity limit of the extract determined using the MTT method adapted from (Arullappan et al. 2013) with a slight modification. The modifications were about using SDS as a stopper of the formation of formazan crystals and the time of the incubation process done overnight. The 100 µL of cells with a density of 1x104 cells/wells put into a 96-well microplate and incubated in a 5% CO_2 incubator at 37°C for 24 hours. The plate is passed out from the incubator, and the medium was discarded. The plate was re-incubated in a 5% CO2 incubator at 37 ° C for 24 hours. The absorbance of each well was read on an ELISA reader at a wavelength of 595 nm. The conversion of absorbance to the percentage of living cells (cell viability) using the formula:

Viability cell (%) = ((Measured absorbance - medium absorbance) / (cell absorbance - medium absorbance)) x 100%.

Immunocytochemistry assay

The ICC assay was used to assay the specific protein related to apoptosis. In this study, they were BCl-2, P53, caspase eight and caspase nine protein. The immunocytochemical method refers to (Susidarti et al. 2014) with slight modifications. Two modifications in the cell incubation after treatment were for 24 hours, and in the use of emersion, the liquid was use entelan.

Data analysis

The IC_{50} values of every sample were calculated using SPSS 16 (Probit/Logit). The differences of IC_{50} values and content of bioactive compounds between each extract were analyzed statistically by analyzing variance (ANOVA) using the SAS ver. 9.1.3. If there is a significant effect of treatment with control, the test is continued with the Tukey test at a test level of 5%. All experiments conducted in five replicates.

RESULTS AND DISCUSSION

Content of bioactive compounds

In this study, the bioactive compounds analyzed were quercetin and kaempferol as a member of the phenolic group and artemisinin as a member of the terpene group of secondary metabolites (Figure 1.). The determination of bioactive compounds using HPLC. HPLC is a chromatographic technique that can be used to identify, measure and purify the individual components (Piana et al. 2013). We used this technique because of its versatility for estimating secondary metabolites in plants (Boligon and Athayde 2014) with the advantages of a fast, sensitive, and accurate analysis process (Martin et al. 2005, Mahendra et al. 2011). In all types of *A. china* analyzed, the content of the three bioactive compounds in the ethyl acetate extracts was higher than the hexane extract. The two highest levels of the three compounds were ethyl acetate extract of J and KJT genotypes that were quercetin at about 0.40-0.55, kaemferol 0.50-0.60, artemisinin 12.0-14.0 µg/mL of extract. The ethyl acetate extract of M and TWN genotypes had lower levels than J and KJT genotypes: quercetin at about 0.25-0.35, kaemferol 0.50-0.60 artemisinin 3.35-4.45 µg/mL of extract.

The ethyl acetate extract contained the three bioactive compounds measured were significantly higher than hexane extract, both in wild or polyploid genotype. The highest content was in the KJT and J genotypes and then TWN and M.

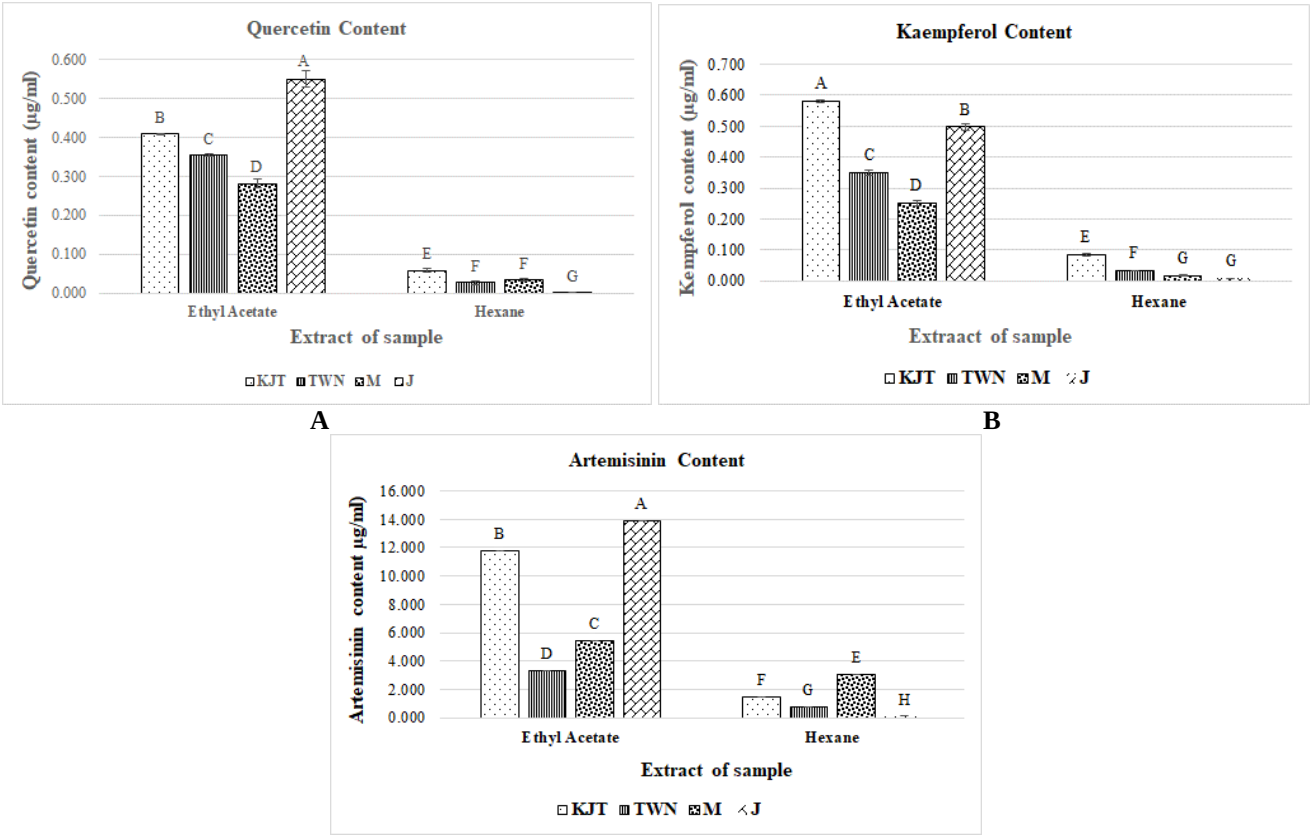

Figure 1. The bioactive content of hexane and ethyl acetate extract of wild and polyploidy type of *Artemisia cina*. A. Quercetin content; B. Kaempferol content; C. Artemisinin content. Note: The value represents the mean (n=3); All mean values were analyzed using one-way ANOVA and followed by the Duncan test to compare the mean difference between samples ($p > 0.05$). The different letters showed significantly different value

Cytotoxic assay

The MTT assay is based on the conversion of MTT into formazan crystals by living cells. It is identical to the activity of mitochondrial. The mitochondrial activity of cell populations is related to the number of viable cells, so this assay is used to measure anticancer agents' in vitro cytotoxic effects on cell lines (Meerloo et al. 2011). The cytotoxicity of all extracts expressed as IC_{50} value. All of the extracts showed the IC_{50} value was more than 200 µg/mL on both cancer cells tested (Table 1.). The IC_{50} value of hexane extracts on WiDr colon cancer cell lines was about 400 µg/mL (KJT and J) and 600 µg/mL (TWN and M) HTB 183. They were around 500-700 µg/mL. On about ethyl acetate extract, based on the IC_{50} value on both cancer cell lines, the ethyl acetate extracts of TWN and M (200-300 µg/mL) were more toxic than hexane extracts, while KJT and J extract were not (300-500 µg/mL).

It appears that the ethyl acetate extract of the TWN and M genotypes can inhibit cancer cell growth more substantially than the others. Both extracts showed IC_{50} value was about 300 µg/mL, while other extracts had IC_{50} more than this, even until about 700 µg/mL.

Immunocytochemistry assay

Immunocytochemistry is a very productive method to identify specific proteins in tissues and cells—the method usually used in biomedical research. To show the location of the test protein correctly and consistently, a control is used as a comparison (Poojan et al. 2018)). This study determined the protein expression related to cell apoptosis that was BCl-2 p53, caspase-8, and caspase-9. Based on the trend in Figure 1, the assay of a marker protein of apoptosis performed on both the extract. As can be seen, M and TWN extracts induced more potent antiproliferative effects in WiDr and HTB-183 cell lines and induced the caspase enzyme activity (Fig. 1 – 3).

Discussion

Herbal medicinal are usually used as sources of agents for traditional medicines for thousands of years. In recent years, natural products and their derivate became beneficial alternative therapy (Akillilar et al. 2018). Artemisia is one of the well-known plants for its antimicrobial, insecticidal, antioxidant, and antimalarial ability. At first, this plant was widely known for its ability to treat malaria, with the bioactive compound artemisinin, but recently the ability of the Artemisia plant as an anticancer agent has been widely studied. Many studies have shown that these bioactive compounds in Artemisia have antimicrobial, antimalarial, antioxidant, anthelmintic and anticancer effects (Hussain et al. 2017). This study was designed to compare the genotype of diploid and polyploidy of *A. cina* in inhibiting the growth of cancer cells WiDr and HTB-183 through cytotoxicity assay by the MTT methods and mechanisms of apoptosis induction by the ICC assay.

Table 1. The cytotoxicity of *Artemisia cina* extracts on the cancer cell lines

Genotype/ Extract	The IC_{50} value of extract on the cancer cell line (µg/mL)			
	WiDr colon cancer		HTB-183 lung cancer	
	Hexane	Ethyl acetate	Hexane	Ethyl acetate
TWIN	679.6 ± 45.1 AB	**292.3 ± 4.0** E	747.2 ± 111.5 AB	**298.5 ± 34.5** E
KJT	481.8 ± 43.6 CD	434.0 ± 30.1 D	556.3 ± 93.3 C	505.7 ± 91.2 CD
J	434.6 ± 102.9 D	325.2 ± 9.9 E	654.4 ± 22.6 B	471.7 ± 34.1 CD
M	662.1 ± 9.6 B	**229.5 ± 19.9** E	768.4 ± 38.0 A	**317.2 ± 23.4** E

Note: The value represents the mean ± standard deviation (SD) (n=3); Mean values in the same column and row having different letters differ significantly were analyzed using one-way ANOVA and followed by the Duncan test to compare the mean difference between samples ($p > 0.05$)

In this study, each genotype extracted with two kinds of solvent was hexane and ethyl acetate. The polarity of ethyl acetate (0.228; medium polarity) is higher than hexane (0.009; a-polar). The phytochemical ability of an extract depends on the bioactive compounds in an extract. The type of bioactive compound carried in an extract is closely related to the solvent used to extract the sample (Dai and Mumper 2010). It means that hexane and ethyl acetate will extract the different bioactive compounds of extract, giving the different cytotoxicity on both cancer cell lines. It is seen in the results of this study, the levels of the compounds assayed were different between both extracts. The content of the compounds in ethyl acetate extract was higher than that of hexane extract. It resulted in different cytotoxic strengths of the extract. Based on the result of statistical analysis of IC_{50} value (Table 1), in general, the results showed the cytotoxic activity of the ethyl acetate extract was more potent than hexane extract on both cancer cell lines, WiDr and HTB-183. Among these, the best antiproliferative ability against the two cancer cells tested was ethyl acetate extract of TWN and M genotype. For the action mechanism study, we used both extracts. Like this result, the antiproliferative ability of several types of Artemisia extracted with various types of solvents including n-hexane, Me-OH, CH_2Cl_2, and EtO-Ac against various cancer cell line (AGS human gastric adenocarcinoma, HT-29 human colon adenocarcinoma, MCF-7 human breast carcinoma, L929 mouse fibroblast) was varied. The types of Artemisia were *A. diffusa*, *A. santolina*, *A. ciniformis*, *A. annua*, *A. biennis*, *A. persica*, *dan A. vulgaris* (Rabe et al. 2011). A study on the *A. Armeniaca* showed that the cytotoxic activity of MeOH extracts and fractions and fractions of n-hexane, CH_2Cl_2, EtOAc, n-BuOH and H_2O on normal K562, HL-60 and J774 cell growth were dose-dependent (Mojarraba et al. 2013). The CH_2Cl_2 and petroleum ether extracts of *A. ciniformis* showed higher cytotoxic on HL-60 and K562 cancer cell lines than EtOAc, EtOH EtOH-water extracts, with IC_{50} value at around 25 µg/mL (Tayarani-Najaran et al. 2014).

The anticancer ability is due to the nature of carcinogenic compounds, both chemical and radiation, which inhibit growth and induce cell death or mediate a common apoptotic pathway. There are several steps to obtain the bioactive compounds from the plant, including drying, grinding, and extraction, and among them, extraction becomes the critical step (Do et al. 2014). One of the influence factors in extraction is solvent (Truong et al. 2019; Zhang et al. 2018). This study used hexane and ethyl acetate as a solvent to take the bioactive content in both polyploidy and wild type of *A. cina*. The three compounds widely recognized as bioactive anticancer agents are quercetin and kaempferol, secondary metabolites of the flavonoid class, and artemisinin is a terpene group. In their review article, Hussain et al. (2017) stated that besides artemisinin, flavonoids, steroids, glycosides, terpenoids, caffeoylquinic acids, acetylenes, coumarins and sterols were also found in Asteraceae. Flavonoids have been demonstrated to have cytotoxic activities toward numerous human cancer cells, whereas they have little or no effect on normal cells (Yuan et al. 2016). Study on ethyl acetate extract of *A. vulgaris* L. and *A. alba* Turra showed that antiproliferative activities increased with time exposure. Both extracts contained phenolic compounds and flavonoids (Jakovljević et al. 2019). The dichloromethane fraction of ethanol eluate of *A. sacrorum* Ledeb showed the most vigorous cytotoxic activities on HepG2, HT-29, and MCF-7 cells EC_{50} values 122.35, 49.76 and 28.51 µg/mL, respectively. One of the compounds of was flavonoid (Piao et al. 2012). The methanol extracts of eight Artemisia species showed the most substantial antiproliferative effect on MCF-7 cells and induced apoptosis via an ER-related pathway at 200 ppm. These eight species were *A. stolonifera*, *A. selengensis*, *A. japonica*, *A. Montana*, *A. capillaris*, *A. sylvatica*, *A. keiskeana*, and *A. scoparia* (Choi and Kim 2013). In the study, ethyl acetate extracts of TWN and M contained quercetin, kaempferol, and artemisinin smaller than KJT and J, but their cytotoxic ability against the two cancer cell lines was more remarkable. The three compounds may cause cytotoxic ability, but there are other compounds that we did not measure in this study. Jakovljević et al. (2019) reported that an ethyl acetate extract of *A. vulgaris* and *A. alba*, which showed the antiproliferative activities, contained phenolic compounds that included phenolic acids (gallic, p-coumaric, vanillic, and ferulic acids), beside flavonoids included rutin, myricetin, luteolin, quercetin, and apigenin. Wang et al. (2012) stated that there are various anticancer compounds, including kaempferol and triterpenoids.

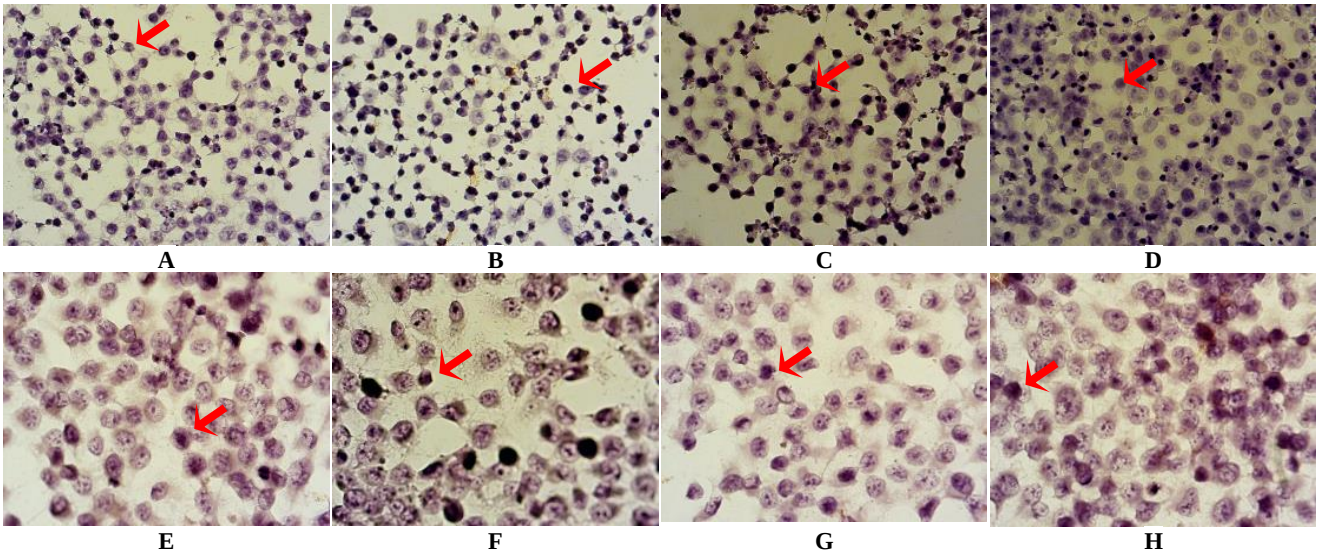

Figure 1. The histology appearance of WiDr and HTB-183 cancer cell line using a light microscope at 400x magnification by treating extract of diploid (TWN) and polyploidy (M) of *Artemisia cina* on apoptosis proteins included Bcl-2, p53, caspase-8, and caspase-9. A. WiDr cell on Bcl-2 expression without treatment; B. WiDr cell on p53 expression with M extract treatment; C. WiDr cell on caspase-8 expression with TWN extract treatment; D. WiDr cell on caspase-9 expression with M extract treatment. E. HTB-183 on Bcl-2 expression without treatment; F. HTB-183 on p53 expression with M extract treatment; G. HTB-183 cell on caspase-8 expression with M extract treatment; H. HTB-183 cell on caspase-9 expression without treatment. Note: The red arrow represents the positive assay.

The excellence of therapeutic to modulate cell life or death is the most sought-after potential. Therefore, the center point of many research focus is on the study of the cell cycle and apoptosis (Elmore 2007). Therefore, the most anticancer drug worked by apoptosis mechanism. Most signal transduction pathways that play a role in the apoptotic process include intrinsic and extrinsic factors via mitochondrial and apoptotic receptors. Proteins such as p53, Bcl-2, Bax, and caspase, are very influential on the apoptosis process. 7, 8, and 9 (Alenzi et al. 2010). Based on that issue, the next step detected the anticancer mechanism's mode by detecting the apoptosis marker protein included BCl-2, P53, caspase-8 and caspase-9, to the most toxic genotype. Bcl-2, an anti-apoptosis protein, is an important signal factor of apoptosis that promotes cancer cell growth (Motadi et al. 2020). In this study, the Bcl-2 expression levels of both cancer cell lines decreased on the treatment of M and TWN extracts. In the HTB-183 cell lines, the M extract decreased to 40%, while TWN extracts until 54%. On the other hand, the M and TWN extract decreased the Bcl-2 expression levels by only 9% and 23% of WiDr cell lines. It appears that the HTB-183 cell lines seem more susceptible to both extracts than WiDr. It also looks at the expression of p53. Mutations of p53 are among the most common genetic abnormalities in human cancers (Karak 2018). In this study, the treatment of M and TWN extracts increased the p53 expression levels at around 100% on HTB-183 cell lines, while on WiDr cell lines at 66% and 39%, respectively. The p53 protein plays a major role in apoptosis induction, and it correlated with increasing caspase activity (Motadi et al. 2020). The occurrence of changes in the expression of p53, a suppressor gene, can trigger the growth of cells into cancer cells, so the search for anticancer agents that can suppress p53 expression is a good potential for the search for new cancer drugs (Astirin et al. 2009). Karak (2018) concluded that flavonoid-induced kinase modulation correlated with apoptosis and proliferation of cells and down-regulation of p53 protein. These results supported the statement of Dhutie et al. (2000) that one of the anticancer mechanisms of flavonoids by down-regulation of p53 protein, cell cycle arrest, inhibition of tyrosine kinase, heat shock proteins inhibition, the binding capacity of estrogen receptor, and Ras proteins inhibition. The study of *Tulbaghia violacea* on some cancer cell lines (HeLa, ME-180, MDA-MBA-231, and MCF-7) showed increased p53 expression until 40% on HeLa and 33% on ME180in treated cells (Astirin et al. 2009). The study on methanol extract of flower and stem of H. *platytaenium* showed that both extracts inhibit T47-D cell lines cancer cell growth but not on MDA-MB-231 cells (Akillilar et al. 2018). From both results, it appears that the extract M and TWN induced the decrease of expression of Bcl-2, a protein marker of cancer incidence followed by increased expression of protein p53, an apoptosis induction. The study on *Cleome droserifolia* showed the methanol extract induced antiproliferative of MCF-7 cells line more than 80% and significantly induced the activity of caspase-8 and caspase-9 (Panicker et al. 2020). Artemisinin act as an anticancer in many mechanisms that induced cell death that induces cell cycle arrest, inhibit angiogenesis and impede cancer metastasis and invasion (Ho et al. 2014; Ng et al. 2014). Many researchers conclude that artemisinin-induced cancer cell death by apoptosis is a primary mechanism (Wong et al. 2017). It said that artemisinin induced apoptosis by activating the release of both caspase 3/9and caspase-8 and cytochrome C or manipulating the expression of anti-apoptotic proteins such as Bcl-2, Bid, and Bak.

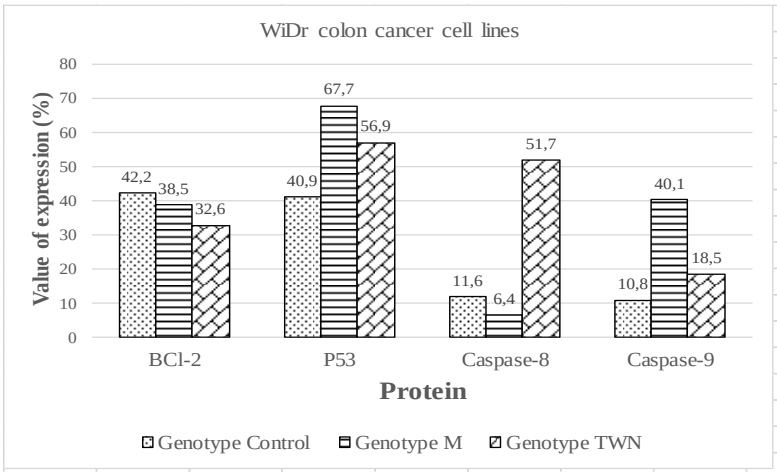

Figure 2. The expression of Bcl-2, p53, caspase-8, and caspase-9 on WiDr colon cancer cell lines treated by ethyl acetate extract of TWN and M, an assay using immunocytochemistry method

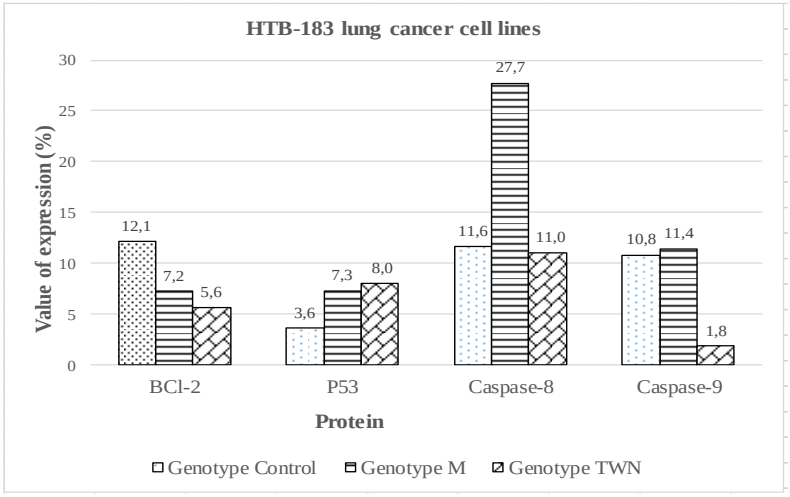

Figure 3. The expression of Bcl-2, p53, caspase-8, and caspase-9 on HTB 183 lung cancer cell lines treated by ethyl acetate extract of TWN and M, an assay using immunocytochemistry method.

In this study, both extracts induced apoptosis cascade in both test cells. In HTB-183 cells, M extracts induced caspase-8 expression up to 1.4 times compared to control without M, while caspase-9 was only 0.6 times. In WiDr cells, M extracts induced caspase-9 expression up to 2.7 times compared to control without M, whereas TWN extract induced caspase-8 expression up to 3.5 times but caspase-9 only 0.7 times. Another researcher reported that artesunate, the artemisinin derivate showed activated caspase-3, -8, and -9 of MCF-7 cell lines after 24 hours of incubation (Motadi et al. 2020; Jamalzadeh et al. 2017). It supports this study that *Artemisia* contains bioactive compounds which can be anticancer agents through mechanical induction of apoptosis. This study supports the study that *Artemisia* contains bioactive compounds that can be anticancer agents through mechanical induction of apoptosis. This study did not assay the caspase-3 expression because if there were caspase-8 or caspase-9 expression, then caspase-3 will be expressed. The TWN extract induced an extrinsic pathway only on both cancer cell lines, while M extracts induced both intrinsic and extrinsic but only on WiDr cancer cell lines.

Thus, the results confirmed *A. cina* potential as an anticancer agent, and we could study another cancer cell. Further, the anticancer activity in vivo assay needs to be done.

In conclusion, from all of the above elaboration, it is concluded that the content of quercetin, kaemferol and artemisinin on ethyl acetate extract higher than hexane extract. The ethyl acetate extract of the TWN and M genotypes, with IC_{50} value, was about 200-300 µg/mL, which can inhibit cancer cell growth more substantially than those with IC_{50} until about 500 700 µg/mL. The ethyl acetate extract of the TWN induced apoptosis of HTB-183 cell lines through caspase-8 and caspase-9 pathways, but ethyl acetate extract of the M induced caspase-9 pathway only.

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