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Research article

Combination treatment of *Chromolaena odorata* ethanolic extract with cisplatin against breast cancer cell lines MDA-MB-231 and MCF-7

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ABSTRACT

Many cancer patients are using complementary and alternative medicines (CAM) in combination with their conventional therapy, more than 72% of them do not inform their physician about CAM use. With the use of CAM in combination with conventional chemotherapeutics, there is an increasing risk for unwanted interactions. *Chromolaena odorata* locally known as 'pokok kapal terbang' and traditionally used for wound healing. However, several studies have been done on cytotoxicity of *C. odorata* against various type of cell lines. Thus, in this study, interaction between cisplatin and *C. odorata* treatment on breast cancer cell lines was evaluated. *C. odorata* leaves were extracted using maceration method with 70% ethanol and the extraction yield was 2.69% (w/w). MCF-7 and MDA-MB-231 cell lines were treated with *C. odorata* ethanolic extract in combination with cisplatin using MTT assay. Combination index (CI) was calculated and isobologram was constructed to evaluate interactions between plant extract and drug. Combination of cisplatin ethanolic extract of *C. odorata* resulted antagonism with combination index greater than one.

Key words: *Chromolaena odorata*, MCF-7, MDA-MB-231, MTT assay, Cisplatin, Breast cancer, Combination index, Iso-bologram.

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INTRODUCTION

According to World Health Organization (WHO), there are some countries that still rely upon plant-based treatment as their source of medicine and developing countries are utilizing compound originated from naturally sourced for therapeutic purposes (Greenwell & Rahman, 2015). Approximately 49% of drugs either natural products or their derivatives are used in cancer treatment and there are 19 drugs derived from natural compounds between year 2005 until 2010 that have been approved (Mangal et al., 2013). Approximately among 9.6 million deaths globally in 2018, 1 in 6 deaths due to cancer which contribute as second leading cause of death (Plummer et al., 2018). In Malaysia, relative survival (RS) for breast cancer in 5 years duration (2007-2011) was 66.8% while in comparison between ethnicity among female, Chinese had highest RS which is 76.5%, followed by Indian 70.5% and Malay 57.9% (Nureylia et al., 2014). Patients always seek alternative approaches and self-medicine using herbal remedies to treat their cancer, alleviate cancer symptoms or drug toxicities because they might experience side effect and slower response towards conventional chemotherapeutic drugs (Engdal et al., 2009). However, 27% patients who take chemotherapeutic drugs and complementary and alternative medicine (CAM) were estimated to possibly develop interaction between drug and CAM (Fasinu et al., 2012). *mChromolaena odorata* has been nominated as the worst invader species by the International Union for Conservation of Nature but it also has some properties in medicinal purpose (Jumaat et al., 2017). In Malaysia, it is usually called as 'pokok kapal terbang' and preferably used for burns, soft tissue wounds, skin infection and it is also believed to enhance homeostasis and stimulate blood coagulation (Nurul Huda et al., 2004). There are studies noted that the leaves have anti-cancer properties which can be used to kill the cancer cell. For example, methanol extract of *C. odorata* alone has proliferative inhibitory effect on HT-29 cell lines (Adedapo et al., 2016). Cisplatin is the first platinum-containing drug approved by FDA in 1978 (DeVita & Chu, 2008). It binds highly to nucleophilic positions of purines base after being hydrolyzed, forming intra-strand DNA adducts crosslinks then inducing apoptosis by several mechanisms such as increase reactive oxygen species and p53 activation (Siddik, 2003). This study focused more on the combination treatment of cisplatin with *C. odorata* ethanolic extract on breast cancer cell lines MCF-7 and MDA-MB-231 in order to evaluate its interaction.

MATERIALS AND METHODS

Plant extraction

Powdered dry leaves of *C. odorata* was obtained and verified by Forest Research Institute Malaysia (FRIM). Ethanol was used as solvent to extract *C. odorata*. Briefly, maceration extraction method with sample to solvent ratio of 1:10 (w/v) was prepared. Plant leaves powder was weighed at 30g and mixed with 300ml ethanol into a flask. The mixture was mixed well and left overnight and filtered the next

day. The extract was evaporated using rotary evaporator, weighed and the extraction yield was calculated using equation (1).

Percentage extraction yield = (mass of extract / mass of sample) × 100 (1)

Cell culture

Two breast cancer cell lines was used in this study namely MDA-MB-231 and MCF-7. The cancer cell line was gained from our laboratory depository. The cell lines were grown and maintained in DMEM medium supplemented with 10% FBS and 1% penicillin streptomycin. The cells then were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell culture medium was changed twice a week.

MTT Assay

MTT assay was conducted following the method described previously with modifications (Abd Mutalib & Abd Latip, 2019). $5x10^3$cells per well was seeded and culture was incubated for 24 hours. 90% confluenced cells in 96 well plate was treated with 100ul of extracts at different concentrations (0-2.5 mg/ml). After 24 hours incubation, 20μL MTT solution (5mg/mL in PBS) was added into each well. After incubated for 4 hours, DMSO was added 50µL per well. Then absorbance value was measured at 570nm. This assay was repeated with cisplatin treatment at different concentrations. The assay was also repeated with combination of extract and cisplatin on the breast cancer cell lines at different ratios. IC50 and Combination Index (CI) values were calculated and isobolograms were constructed. Equation 2 shows the formula to calculate CI values. All statistical analysis was done using IBM SPSS Statistics 25 software.

CI= (IC50extract combination / IC50extract single) + (IC50drug combination / IC50drug single) (2)

RESULTS AND DISCUSSION

Preparation of ethanolic extract involved maceration method and solvent removal using rotary evaporator at 40˚C for 3-4 hours to produce crude ethanolic extract of *C. odorata.* The yield of extraction calculated is 2.69%. The percentage yield is lower than previous study conducted which is 3.8% (Stanley et al., 2014). However, there are slightly modification of extraction of the previous study such as they soaked for 72 hours instead of 24 hours. Extraction efficiency from plants is related to time of extraction, solvent type, particle sizes of the plant and plant material to solvent ratio (Dhawan & Gupta, 2016).

Anti-proliferative activity of the extract was screened on four different cell lines as shown in Figure 1. It is found that the *C. odorata* extract have various potency of anti-proliferative activity against each cell lines. The lower value of IC_{50}, indicates the higher ability of extract to exert its inhibition activity. The anti-proliferative activity of the extract is the highest against MCF 7 (oestrogen positive receptor breast cancer) and the lowest against CRL 2522 (skin fibroblast) cell line. The ethanolic extract of *C. odorata* against all the cell lines does not exhibit good anti-proliferative activity as based on American National Cancer Institute as the IC_{50} limit to consider a crude extract promising for further purification should be lower than 30µg/ml (Alsabri et al., 2013). However, when compared between cell lines in this study, ethanolic extract of *C. odorata* showed selective inhibitory effect between normal cell line and cancer cell line. Previous study also showed that ethanolic extract of leaves exhibit both cytotoxic and anticlonogenic actions against a variety of cancer cell lines such as Cal51, MCF7 and MDAMB-468 due to certain chemical compounds isolated from the extract such as 20-hydroxy-4,40,50,60-tetramethoxychalcone by inducing apoptosis (Kouamé et al., 2013) .

Figure 1: IC_{50} (mean ± SEM) of anti-proliferative activity of *C. odorata* ethanolic extract against MCF-7 (oestrogen positive receptor breast cancer), MDA-MB-468 (oestrogen negative receptor breast cancer), WRL-68 (normal hepatocyte), and CRL-2522 (skin fibroblast). Different letters denote significant difference (p-value<0.05, ANOVA post-hoc Tukey).

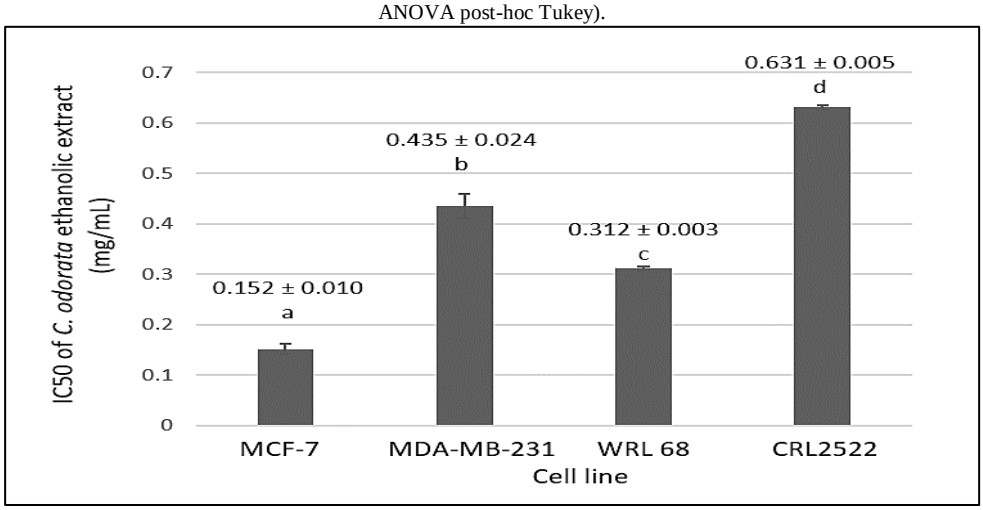


Figure 2 and Figure 3 illustrates anti-proliferative activity of *C. odorata* ethanolic extract and cisplatin respectively on MCF-7 and MDA-MB-231 cell lines. Both treatment showed inhibition in dose dependent manner. MCF-7 cell line demonstrate higher sensitivity towards both treatment in comparison to MDA-MB-231. IC_{50}, IC_{25}, IC_{15}, IC_{10} values of *C. odorata* ethanolic extract and cisplatin on MCF-7 and MDA-MB-231 cell lines are listed in Table 1. These values show MDA-MB-231 is less susceptible to be inhibited by both extract and cisplatin treatment at various concentrations. This finding is similar to previous study which reported cisplatin exerts higher inhibition in MCF-7 compared to MDA-MB-231 (Wen et al., 2013).

Figure 2: Percentage inhibition of *C. odorata* ethanolic extract against breast cancer cell lines (MCF 7 and MDA-MB-231). Bars with different letters represent that there is significant difference between treatment of different concentrations of extract with untreated (p<0.05, Independent Student t-test).

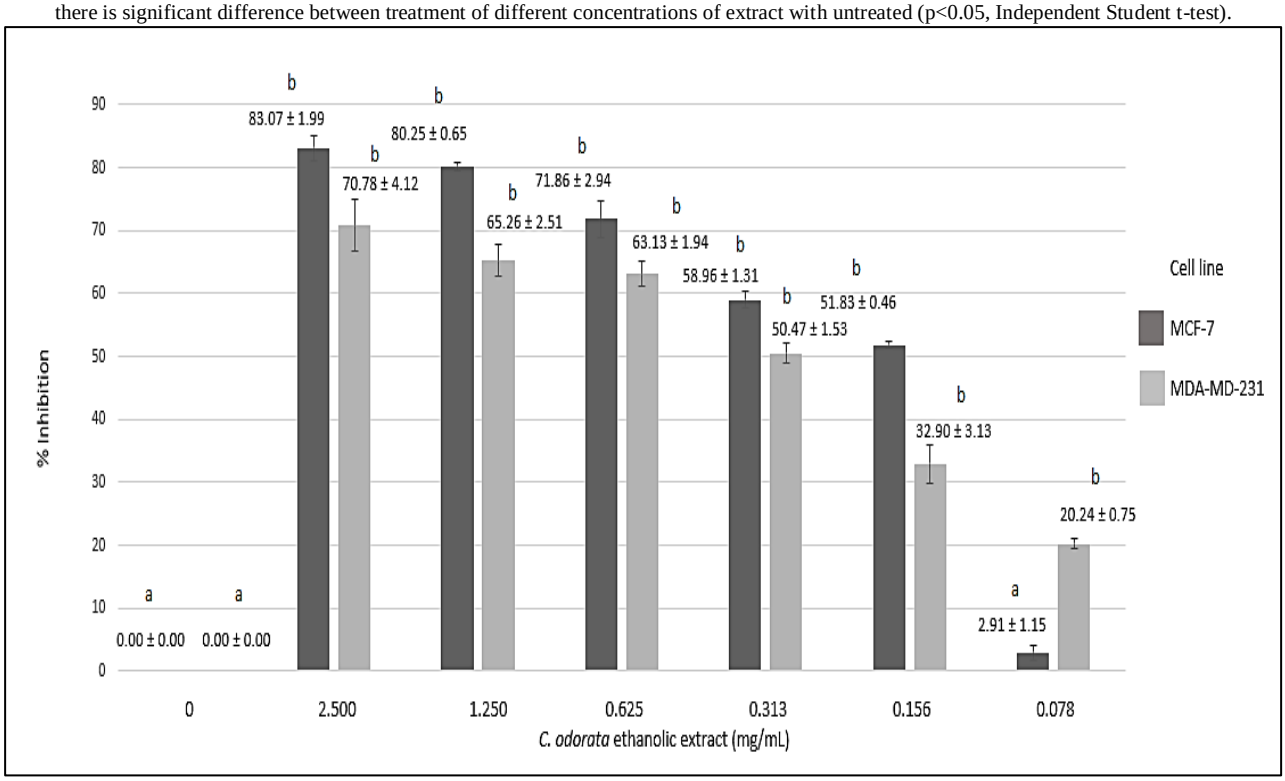


Figure 3: Percentage inhibition of cisplatin against breast cancer cell lines (MCF 7 and MDA-MB-231). Bars with different letters represent that there is significant difference between treatment of different concentrations of extract with untreated (p<0.05, Independent Student t-test).

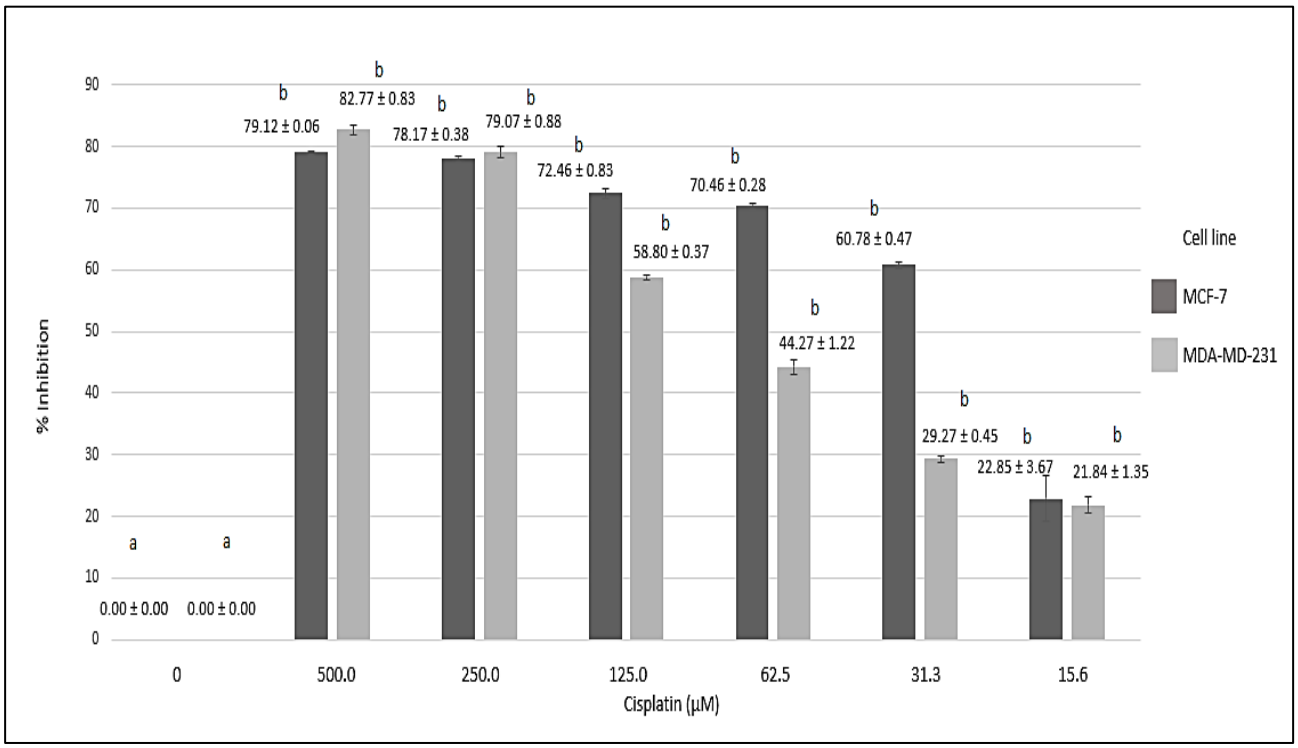


Table 1: IC_{50}, IC_{25}, IC_{15}, IC_{10} values of *C. odorata* ethanolic extract and cisplatin on MCF-7 and MDA-MB-231 cell lines.

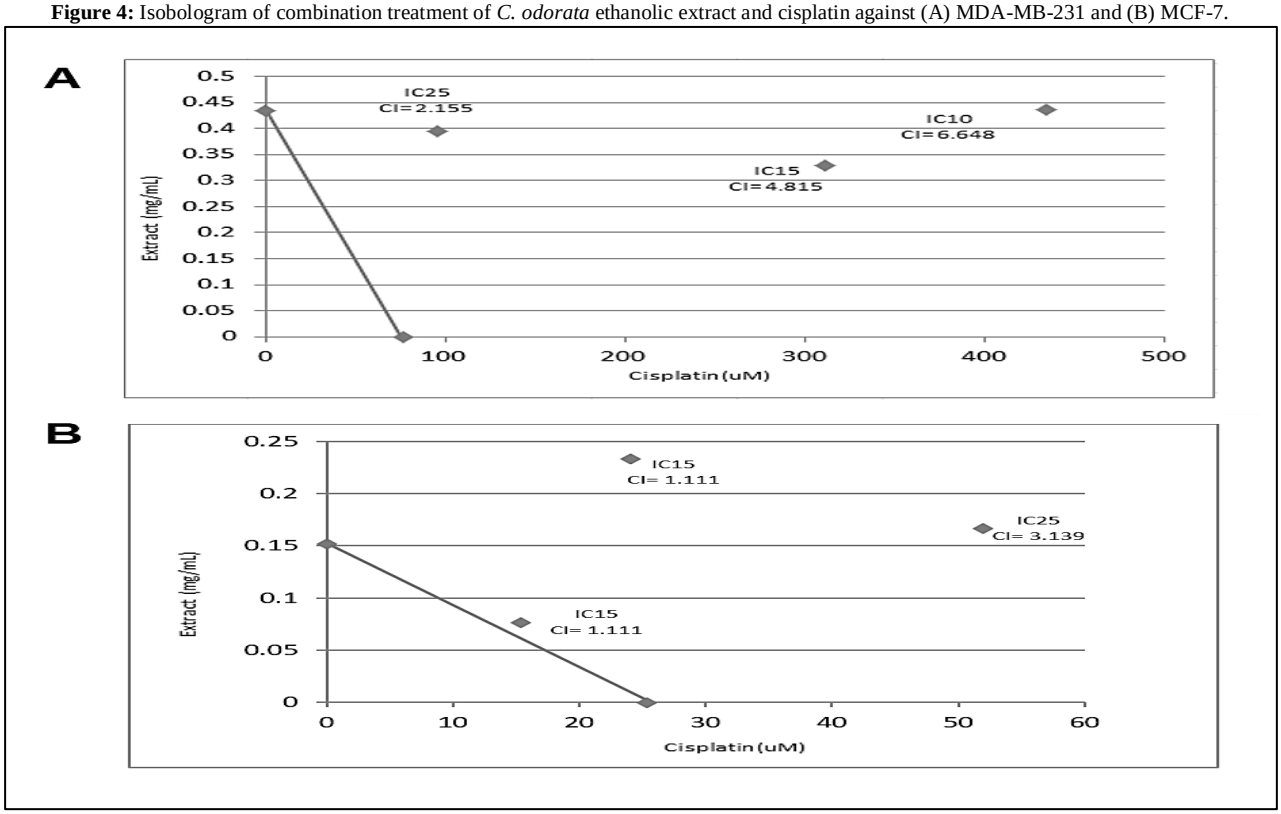
Treatment	IC_{50} (mean ± SEM)		IC_{25} (mean ± SEM)		IC_{15} (mean ± SEM)		IC_{10} (mean ± SEM)	
	MCF-7	MDA-MB- 231	MCF-7	MDA-MB- 231	MCF-7	MDA-MB- 231	MCF-7	MDA-MB-231
C. odorata ethanolic extract (mg/ml)	0.152 ± 0.010	0.435 ± 0.024	0.107 ± 0.010	0.080 ± 0.012	0.093 ± 0.010	0.041 ± 0.009	0.086 ± 0.010	0.030 ± 0.007
Cisplatin (µM)	25.419 ± 0.812	76.986 ± 0.824	16.195 ± 1.015	21.097 ± 0.112	13.626 ± 0.925	12.571 ± 0.042	12.516 ± 0.866	9.703 ± 0.024

Although, ethanol extract of this plant has not shown promising anti-proliferative effect, with the use of in combination with conventional chemotherapeutics drug, there is possible risk for unwanted interactions, especially because of the narrow therapeutic index of most oncolytic drugs (Meijerman et al., 2006). Due to increasing findings regarding the anti-proliferative activity of this plant in previous studies, this plant potentially be taken by cancer patients as complimentary treatment. Thus, the effect of combination treatment of *C. odorata* ethanolic extract and cisplatin on breast cancer cell lines. Combination index of co-treatment of *C. odorata* ethanolic extract and cisplatin on MCF-7 and MDA-MB-231 cell lines at different ratios were calculated and tabulated in Table 2. All combination treatment on both cell lines resulted in values of combination index greater than one. This indicates antagonistic interactions between both treatments. The interaction further illustrated in Figure 4 showing isobologram with all plots located above the additivity line that is also an indication for antagonism.

Table 2: Combination Index

Combination Treatment CP : Cisplatin CO : *C. odorata*	**CELL LINE**	**Combination Index (CI)**	**Description**
CP + CO IC10	MCF-7	2.481	Antagonism
	MDA-MB-231	6.648	Antagonism
CP + CO IC15	MCF-7	1.111	Antagonism
	MDA-MB-231	4.815	Antagonism
CP + CO IC25	MCF-7	3.139	Antagonism
	MDA-MB-231	2.155	Antagonism

Figure 4: Isobologram of combination treatment of *C. odorata* ethanolic extract and cisplatin against (A) MDA-MB-231 and (B) MCF-7.



Due to the antagonistic interaction by *C.odorata* extract, mechanism of action of cisplatin might be disrupted. Previous report explained that cisplatin exerts its cytotoxicity by forming DNA adducts and increase reactive oxygen species (ROS) due mitochondrial DNA damage which both mechanisms lead to cell death (Marullo et al., 2013). The formation of ROS depends on the concentration of cis-diamminedichloro platinum (II) and the duration of exposure which induce apoptosis by intrinsic or extrinsic pathway (Dasari & Bernard Tchounwou, 2014).

However, compounds that impede ROS generation could prevent cisplatin-induced apoptosis (Choi et al., 2015). Data from previous study indicated ethanol extract *C. odorata* leaf contains flavonoids, terpenoids, phenols, saponins, cardiac glycosides and alkaloids (C E C et al., 2017). Another study demonstrated that the crude ethanol extract of *C. odorata* contains phenolic acids (protocatechuic, p-hydroxybenzoic, p-coumaric, ferulic and vanillic acids) and complex mixtures of lipophilic flavonoid aglycones (flavanones, flavanols, flavones and chalcones) (Vijayaraghavan et al., 2017). Hence, most of the phytochemical compounds found in *C. odorata* extract are antioxidants.

It has been proposed that the antioxidant properties of phenolic compounds have ability to scavenging and suppressing ROS/RNS generation by inhibit some enzymes or chelating metals that involved in free radical synthesis or enhance antioxidant defense (Kocyigit et al., 2018). Thus, this mechanism might interfere cisplatin ability to kill cancer cell through ROS. Therefore, further study needs to be done to elucidate either *C. odorata* extract significantly suppress ROS production or there are other mechanism involved contributing to its antagonistic interaction posed towards cisplatin.

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