

## An Extract of *Zingiber officinale* and *Piper retrofractum* Combination and Its Effect to Cancer Cell Line

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### Abstract

Chemotherapy may emerge side-effect since it may treat inconveniently the synthesis of nucleic acids and proteins, both cancer cells or normal cells. Plants as a cancer therapy were expected to reduce this toxicity and side effects. Plants which used empirically for cancer therapy was *Zingiber officinale* cv. Rubrum and *Piper retrofractum*. This study was conducted to examine the cytotoxic activity of ethanolic extract combination of two plants in HeLa and T47D cell lines. *Zingiber officinale* cv. Rubrum, *Piper retrofractum* and mixture (1:1) powdered then macerated with 96% ethanol for 3 x 24 hours. Identification of the constituent that had potential anticancer effect was used TLC with silica GF<sub>254</sub> as stationary phase, cytotoxic activity was examined by yellow MTT assay, then analyzed using probit. Apoptotic assay was performed by immunofluorescence method, using fluorochromes ethidium bromide and acridine orange. The result showed that *Zingiber officinale* cv. Rubrum contains terpenoids, while *Piper retrofractum* contains alkaloids substance. The mixture showed cytotoxic activity against HeLa and T47D cell with IC<sub>50</sub> 33 and 53 µg/mL respectively. The extract caused cytotoxic effect through apoptotic mechanism.

**Keywords:** *Zingiber officinale* cv. Rubrum, *Piper retrofractum*, cytotoxic, HeLa cells, T47D cells

### INTRODUCTION

World Health Organization (WHO) provides an illustration that 12% of all deaths in the world caused by cancer. Cervical carcinoma is the leading cause of cancer-related death in women [Suwiyoga, 2007]. Based on data from the Ministry of Health of the Republic of Indonesia in 2005 incidence of cervical carcinoma was second ranked after breast cancer [Anonymous, 2007]. Cancer chemotherapy are include cytostatic drugs, hormones, antihormon, and biological compounds. These drugs cause side effects because its affect to synthesis of nucleic acids and proteins, so the cancer cells and normal cells will be damaged [Stetler and Kleiner, 2001].

Research for cancer drug conducted to discover new drugs for cancer therapy. Indonesia is rich in natural ingredients, particularly plant materials, which are used empirically for cancer, including red ginger (*Zingiber officinale* cv. Rubrum) and java chili (*Piper retrofractum*).

Research has been conducted on red ginger, i.e anti-cancer activity and anti-inflammatory drug on liver cancer [Habib *et al.*, 2008], inhibit the activity of cell growth and modulates secretion angiogenic factor in ovarian cancer cells [Rhode *et al.*, 2007]. Research of Kim *et al.* [2008] showed that the red ginger rhizome have active content as a cytotoxic agent, namely oleoresin consisting of gingerol, paradol, shogaol, zingerone, resin and volatile oil which is a group of terpenoids [Ravindran *et al.*, 2005].

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Research in vitro and in vivo also has been conducted on piper java chili extract. Java chili has been investigated to have antioxidant activity [Jagdale *et al.*, 2009; Wakade *et al.*, 2008], analgesic activity, used to reduce pain in cancer [Febrina and Subarnas, 2006], and toxic effects on cells Myeloma with IC<sub>50</sub> of 55,48 µg/mL [Setyorini, 2007]. As anticancer active content on the piper java fruit are alkaloids [Selvendiran *et al.*, 2003; Pradeep and Kuttan, 2004].

Cancer treatment by combining several plants in Thailand called Pikutbenjakul done by Sakpakdeejaroen and Itharat [2009], reported to have cytotoxic activity Linn Piper chaba, *Zingiber officinale*, and herbal medicine Pikutbenjakul (*Piper chaba* Linn, *Piper sarmentosum* Roxb, *Piper interruptum* Opiz., *Plumbago indica* Linn. and *Zingiber officinale*) against breast adenocarcinoma cells (MCF-7) obtained IC<sub>50</sub> *Piper chaba* Linn, *Zingiber officinale*, and Pikutbenjakul of 35; 31 and 33 µg/mL, respectively.

The p53 gene appears to trigger programmed cell death (apoptosis) as a way of regulating uncontrolled cellular proliferation in the setting of aberrant growth signals [Rugo, 2006]. When cellular stress (e.g. DNA damage) occurs, proapoptotic proteins in the cytosol will be activated. As a result, cytochrome c localized in mitochondria will be released to the cytosol, activated caspase-9 [Fan *et al.*, 2005]. The purpose of this study was to investigate the active constituent of ethanolic extract of ginger rhizome and java chili and its cytotoxic and apoptosis activity on HeLa and T47D cells.

## METHODS

### Plant materials, chemicals, cell line and culture

*Z.offinale* rhizome and *P. Refractum* were purchased on the local market and authenticated in taxonomy laboratory at biology Faculty Jenderal Soedirman University and stored as a voucher specimen in the same faculty. Cisplatin and tamoxifen were obtained from parasite laboratory, Faculty of Medicine, Gadjah Mada University. HeLa and T47D cells were obtained from parasite laboratory, Faculty of Medicine, Gadjah Mada University. HeLa and T47D cells were routinely cultured in RPMI 1640 medium (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA) at 37°C in a 5% CO<sub>2</sub> atmosphere, 3% penicillin- streptomycin and 1% fungison. Subcultures were obtained after

treatment with 0.05% trypsin (Gibco, Auckland) in phosphate buffered saline.

### Preparation of extract combination

Red ginger and java chili washed, cut into pieces, dried and crushed into powder. 400 grams of red ginger rhizome powder, 400 grams of piper java fruit and combination of red ginger: java chili 1:1 (one each 200 gr) were extracted by maceration using 96% ethanol for 3 x 24 hours. The extract is filtered and then evaporated.

### Constituent Identification

The extract then analysis using Thin Layer Chromatography (TLC) with silica gel GF<sub>254</sub> stationary phase and mobile phase toluene-ethanol-acetic acid (8: 2: 1) for terpenoids in red ginger rhizomes and n-hexane-ethyl acetate (3 : 2) for alkaloid in java chili. Extracts from each sample was spotting in TLC plate, eluted with mobile phase, and sprayed with Vanillin-H<sub>2</sub>SO<sub>4</sub> reagent for terpenoids and Dragendorff reagents for alkaloid. The spots were observed using UV light with wavelength 254 nm.

### Preparation of the test material (stock solution)

The ethanol extract of red ginger rhizome, Java piper, and mixtures were weighed 20 mg, then added 100 mL of DMSO as stock solution. The concentration of extract and cisplatin obtained from dilution with RPMI-1640 medium for the cytotoxic and apoptosis test.

### Cell viability assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

For cell viability assay,  $1.5 \times 10^4$  cells/well were plated in 100 µl of RPMI 1640 media. Cells were incubated overnight at 37°C in humidified atmosphere of 5% CO<sub>2</sub> for cells attachment. Extract was added at various concentrations ranging from 500; 250; 125; 62.5; 31.25; 15.63; and 7.81 µg/mL after 24 h incubation. After 24 h incubation, 100 µL MTT (5 mg/mL in PBS) was added to the plate. The resulting MTT-products were determined by measuring the absorbance at 595 nm with ELISA reader. Each point represents the mean of triplicate experiments. Absorbance data then calculated to the standard curve equation of HeLa and T47D cells in order to get the number of living cells in the control cells and the number of living cells in the test cell. Inhibition percentage of each test solution is determined to obtained IC<sub>50</sub> with probit analysis using SPSS (Statistical Product and Service Solutions).

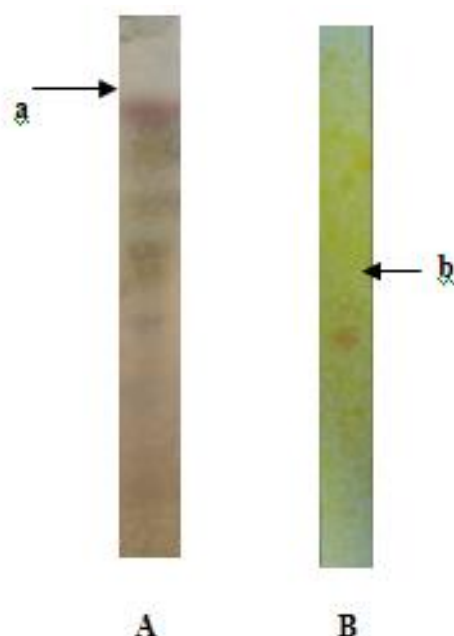
### Determination of Apoptosis

Cells were grown on glass coverslips in tissue culture dishes (Falcon) and were allowed to attach for 24 hr prior to the addition of drug. After the cells were incubated with drug for 24 hours, the coverslips were washed once in phosphate-buffered saline and fixed in object glass. Treated cells were stained with acridine orange and ethidium bromide 5  $\mu$ L and visualized by fluorescence microscopy. Viable (normal, green nuclei), early apoptotic (condensed, green nuclei) and late apoptotic (condensed, red nuclei) cells were observed.

### RESULTS

**The extract had active substance for its anticancer activity**

Stationary phase used is silica gel GF254 plates that have been coated with compounds that can berflouresens at wavelength 254 nm. Mobile phase used was a mixture of toluene-ethanol-acetic acid with a ratio of 8: 2: 1 for identification of terpenoids on red ginger rhizome extracts. From the TLC image of red ginger rhizome extract (Figure 1), after sprayed with vanillin-sulfuric acid reagent and then heated at 1000C for 10 minutes in visible light there was red purple spots. Further observation under UV 254 gained 3 spots. Patches of red ginger rhizome on UV 254 reduction is not too clear. The first spots of purple red indicates that the red ginger rhizome extract contains terpenoids.



**Figure 1. Thin Layer Chromatography. Stationary phase: Gel Silica G<sub>254</sub>, Mobile phase: A. toluene-ethanol-acetic acid (8:2:1) sprayed with Vanillin-H<sub>2</sub>SO<sub>4</sub> reagent (red purple spot-a)), B. n-hexane-ethyl Acetate (3:2) sprayed with dragendorf reagent (brown-orange spot-b)**

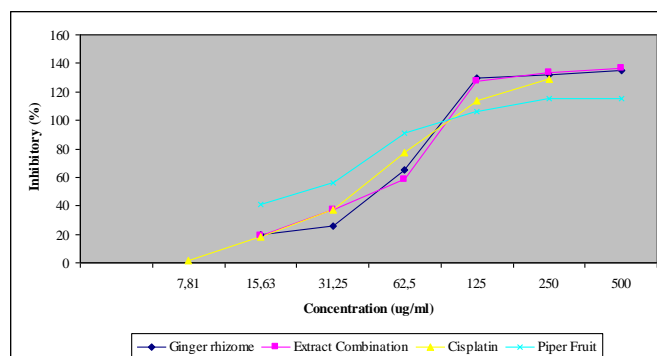
Mobile phase mixture of n-hexane and ethyl acetate with a ratio of 3: 2 is used for identification of alkaloids in the fruit extract of cayenne Java. Observation under UV light 254 seen seven spots and sixth spots appear orange.

### Extract combination had cytotoxic activity on HeLa and T47D Cell Lines

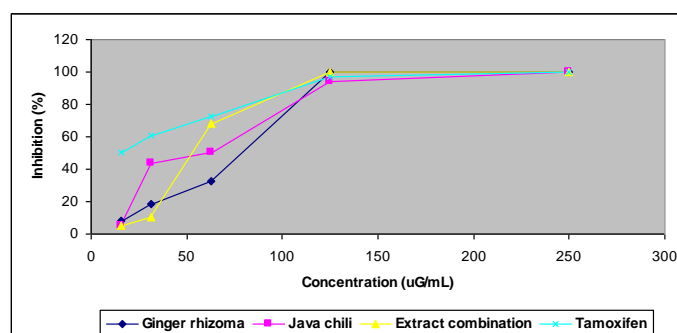
Figure 2a. showed increased concentrations of the test material causes an increase in the percentage inhibition of Hela cell growth. At a concentration of 62.5  $\mu$ g/mL, red ginger rhizome extract, Java piper, and a mixture capable to inhibit the growth

of HeLa cells at 65 %, 58 % and 77 %. Cisplatin has the inhibition on the growth of HeLa cells at a

concentration of 15 ug / mL of 91%.



(a)



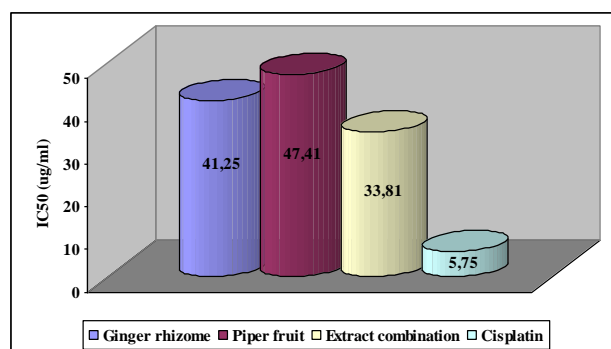
(b)

**Figure 2.** Inhibition rate on HeLa (a) and T47D (b) cell line by ginger rhizome, java chili, extract combination, tamoxifen and cisplatin. HeLa and T47D cells were treated with various doses of ginger rhizome, java chili, extract combination, tamoxifen and cisplatin, incubated for 24 hours at 37°C in humidified 5% CO<sub>2</sub> atmosphere. Cell viability was determined by MTT assay, absorbance was read at 595 nm. Inhibition rate (%) was defined as: ((live cell in the control – live cell in the test group)/live cell in the control) × 100. Standard curve:  $y = 0.00001x + 0.207$  ( $R^2 = 0.927$ ) for HeLa cells and  $y = 0.00001x + 0.3411$  for T47D cells ( $R^2 = 0.9906$ ). Results are average of three independent experiments (mean ± SD).

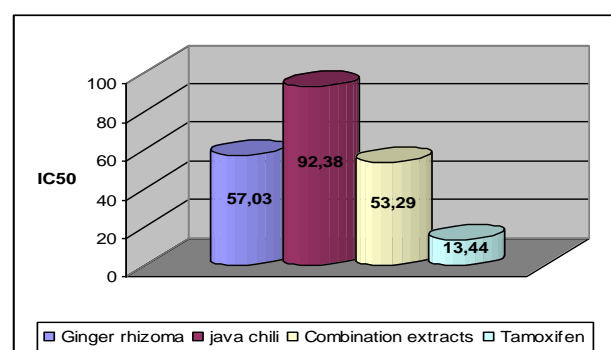
Figure 2b. showed increased concentrations of the test material causes an increase in the percentage inhibition of T47D cell growth. At a concentration of 62.5 ug/mL, red ginger rhizome extract, Java piper, and a mixture capable to inhibit the growth of T47D cells at 32 %, 47 % and 62 %.

Cisplatin has the inhibition on the growth of T47D cells at a concentration of 15 ug/mL of 90 %.

Figure 3. showed that the material has the ability to reduce the growth of cells as shown by the IC<sub>50</sub> value. Mixture of red ginger rhizome extracts and Java chili (1:1) has a IC<sub>50</sub> of 33 ug/mL in Hela cells and 68 ug / mL in T47D cells.



(a)

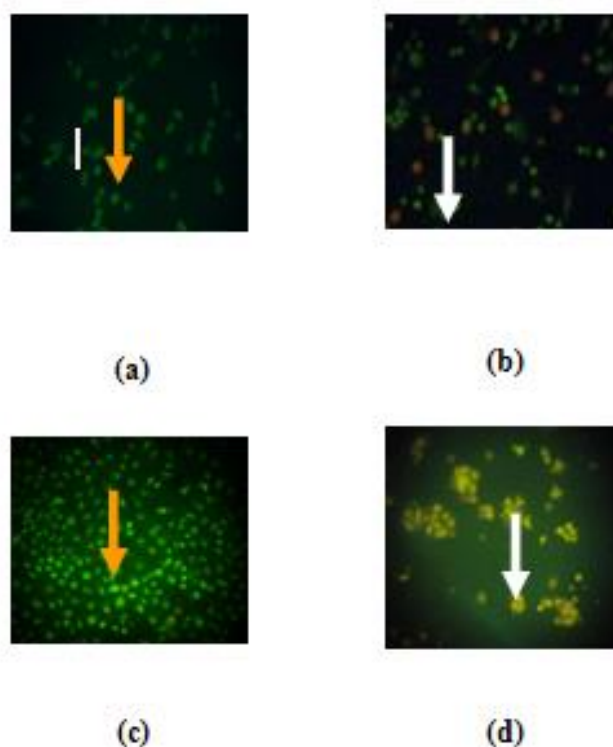


(b)

**Figure 3.** IC<sub>50</sub> of ginger rhizome, java chili, extract combination, tamoxifen and cisplatin on HeLa (a) and T47D (b) cancer cells. Concentrations inhibiting 50% of the cell were determined by probit analysis using SPSS software.

### Extract combination induced apoptosis

Cells treated with extract combination with IC<sub>50</sub> dosis, underwent the classical and colour changes indicate of apoptosis.



**Figure 4.** HeLa (a,b) and T47D (c,d) cells were treated with acridine orange and ethidium bromide. Viable cell (orange arrow), apoptotic cell (white arrow). Cells treated with extract combination (ginger rhizome: java chili = 1:1) with IC<sub>50</sub>, underwent the classical and colour changes indicate of apoptosis.

## DISCUSSION

Identification of chemical constituents of red ginger and java chili extracts conducted to determine the active compounds which efficacious for cancer. The study reported that terpenoids from extracts of red ginger rhizome and alkaloids of java chili had potential effect as anticancer [Shukla *et al.*, 2007; Selvendiran *et al.*, 2003]. Silica gel GF<sub>254</sub> was used as stationary phase. According to Stahl [1985], silica gel has a moisture that significantly affected the separation power. Toluene-ethanol-acetic acid (8: 2: 1) was used as mobile phase for identification of terpenoids on red ginger extract. Toluene (C<sub>7</sub>H<sub>8</sub>) has a dielectric constant 2.3, so will dissolve non polar compounds. Acetic acid was a polar solvent which has a dielectric constant 6.2 may dissolve polar compounds such as inorganic salts and organic compounds. While ethanol has a dielectric constant 24.3, more polar than acetic acid. Oleoresin in red ginger included in the group of

terpenoids that expected to be able to separate with mobile phase.

Results after sprayed with vanillin-sulfuric acid reagent and then heated at 100°C for 10 minutes to accelerate the reaction formation and intensity of the spot color [Rohman, 2009]. In visible light visible there was purple-red spots. Further observation under UV light 254 obtained three spots. According to Stahl [1985], spots of red-violet color spot of red ginger is zingeron.

Mobile phase mixture of n-hexane and ethyl acetate with a ratio of 3: 2 are used for identification alkaloids in extracts java chili. Dielectric constant of n-hexane and ethyl acetate successively are 1.890 and 6.02. N-hexane is more nonpolar than ethyl acetate which is semipolar. The selection phase was based on active compounds namely alkaloids piperine which that are semipolar. Therefore, with similar levels of polarization as this makes the greater separation of substances [Stahl, 1985]. Dragendorff reagent (potassium tetraiodobismutate or BI<sub>3</sub>KI) was a

reagent which bind to the electron on N atom of the alkaloid. Observation under UV light 254 seen seven spots and sixth spots appear orange. According to the theory proposed by Wagner and Bladt [1995] stated that the positive results of alkaloids with Dragendorff reagent spray, brown or orange. With the existence of six orange spots showed that java chili extract contains alkaloids.

Figure 2. showed that the material has a ability to inhibit HeLa and T47D cell growth as shown by the  $IC_{50}$  value. This indicates that the combination has cytotoxic activity against HeLa cells. Meyer *et al.* [1982] declares an extracts said to have anticancer activity if the  $IC_{50}$  value of less than 1000  $\mu\text{g/mL}$  after 24 hours of contact time.  $IC_{50}$  is concentration that can inhibit cell growth by 50% cell line. The smaller the  $IC_{50}$  of a compound the more toxic compound it was [Doyle and Gaffiths, 2000].

Merging or combining several plants in cancer treatment performed to enhance the cytotoxic activity and minimize side effects caused by the use of anticancer drugs [Beinfeld, 2005]. Cytotoxic activity of extract combination against HeLa cells was higher with an  $IC_{50}$  value of 33.807 compared with  $IC_{50}$  values of each extract, i.e 41.249 and 47.409  $\mu\text{g/mL}$  for red ginger rhizome and piper java fruit extracts. This is because of red ginger and piper java fruit has a different mechanism against cancer. Red ginger rhizome could raise natural killer cell activity (NK) to lysis target cells, namely tumor cells and virus-infected cells [Zakaria *et al.*, 1999] and is able to inhibit the activity of NF $\kappa$ B (Nuclear Factor kappa B) through the inhibition of cytokine pro inflammation, so the emergence of inhibit TNF- $\alpha$  which is the cause of the emergence of tumors [Habib *et al.*, 2008; Hudson *et al.*, 2000]. Piperine contained in piper java fruit protect cells from cancer by binding proteins in the mitochondria to trigger apoptosis without harming normal cells through enhanced activity of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase [Selvendiran *et al.*, 2003]. Additionally, piperine may inhibit NF $\kappa$ B thereby preventing the formation of tumors through TNF-, so angiogenesis does not occur [Pradeep and Kuttan, 2004]. Given the different mechanism of action following the combination of plants can enhance the cytotoxic activity.

Red ginger rhizome extracts had  $IC_{50}$  values up to 41.249  $\mu\text{g/mL}$  less than the piper java fruit extract of chili java 47.409  $\mu\text{g/mL}$ . Differences in mechanism of action against cancer cells affect the cytotoxic activity. Research carried out by Rhode *et al.* [2007] showed that the red ginger can inhibit

cell growth and modulates secretion angiogenic factor in ovarian cancer cells. Therefore, a potential red ginger in the treatment and prevention of ovarian cancer. Another study carried out on liver cancer and metastases by inhibiting activation of CD8 + T cells [Habib *et al.*, 2008; Suzuki *et al.*, 1997]. Red ginger able to minimize the side effects of cancer drugs such as nausea and vomiting [Ernst and Pittler, 2000].

Piper java fruit extract in this study have cytotoxic activity against HeLa cells with  $IC_{50}$  of 47.409  $\mu\text{g/mL}$ . This showed that 96% ethanol extract of piper java fruit has cytotoxic activity against HeLa cells was better than the 70% ethanol extract of chilies java [Suhartatik, 2008]. In addition, cytotoxic activity against HeLa cell of piper java fruit extracts better than against myeloma cells. This is shown by  $IC_{50}$  values of piper java fruit against HeLa cells are smaller than the myeloma cells that is 46.246  $\mu\text{g/mL}$  and 55.48  $\mu\text{g/mL}$ . This difference in the chances of having the target compounds and causes of action of cancer cells in HeLa cells and myeloma cells. The study by Choi *et al.* [2009] showed that the piper fruit can reduce the risk of cisplatin resistance of cancer cells by induction of apoptosis via heme oxygenase-1 (HO-1). Cisplatin has the smallest  $IC_{50}$  value of 5.745  $\mu\text{g/mL}$ . It showed better cytotoxic activity of cisplatin against HeLa cells. Based on the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, cisplatin is the first-line monotherapy in the treatment of cervical cancer [Teng *et al.*, 2004].

Ginger extract at increasing concentrations induced apoptosis dose dependently in colon cancer cells [Abdullah *et al.*, 2010]. [6]-gingerol associated with the modulation of p53 and involvement of mitochondrial signaling pathway in B[a]P-induced mouse skin tumorigenesis [Nigam *et al.*, 2009].

Java chilli fruit extract protects cells from cancer by binding proteins on cancer cell mitochondria to trigger apoptosis without damaging the surrounding cells through increased activity of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase [Selvendiran *et al.*, 2003]. In addition, chilies java can inhibit NF $\kappa$ B, inhibit tumor formation by TNF- $\alpha$  that resulted in no occurrence of angiogenesis [Pradeep and Kuttan, 2004].

## CONCLUSION

*Zingiber officinale* cv. Rubrum had terpenoids, while *Piper retrofractum* had alkaloids

substance. The mixture showed cytotoxic activity against HeLa and T47D cell with IC<sub>50</sub> 33.807 and 53.289 µg/mL respectively. The extract caused cytotoxic effect through apoptotic mechanism.

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