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# Chemical composition and anti-proliferative properties of flowers of *Clitoria Ternatea*

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**Abstract**

Aqueous and methanol extracts of the flowers of *Clitoria ternatea* (CT), a popularly plant consumed for blue colour in Nasi Kerabu was selected to explore its cytotoxic effect on six types of normal and cancer-origin cell lines. These included the hormone-dependent breast cancer cell line (MCF-7), non-hormone-dependent breast cancer cell line (MDA-MB-231), human ovary cancer cell line (Caov-3), human cervical cancer cell line (Hela), human liver cancer cell line (HepG2) and human foreskin fibroblast cell line (Hs27). The anti-proliferation activities of the extracts were examined by employing colorimetric MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) assay through time periods of 24, 48 and 72 hours. Preliminary results showed that the water extracted of CT had significant effects ($p < 0.05$) against MCF-7 with an $IC_{50}$ value of 175.35 µg/ml. Furthermore, the aqueous and methanolic extracts were investigated by Gas Chromatogram-Mass spectrometry (GC-MS). The GC-MS chromatogram analysis of the water extracted had shown five peaks that represented components in the water extract namely mome inositol (38.7%) and pentanal (14.3%). Fifteen chemical constituents were identified in the methanol extract and the major chemical constituents were mome inositol (33.6%), cyclohexen, 1-methyl-4-(1-methylethylideme)- (7.1%), acetic acid, cyano- (6.5%) and hirsutene (5.7%). Heavy metals tested were at very low levels. The analysis conducted on the flowers provides a strong basis for emphasizing the medicinal and nutritional value of CT.



## Introduction

The World Health Organization (WHO) has declared that total mortality due to cancer will increase to 12 million in 2030 whereas this number was only 7.6 million in 2005 (Farooqui *et al*., 2011). In year 2006, National Cancer Registry had reported about 21,773 cancer cases were diagnosed in Peninsular Malaysia (Zainal *et al*., 2006). Recently, cancer chemoprevention has developed as a major attention for researchers (Kim *et al*., 2010).

Treatment used against cancer such as chemotherapy, radiation, hormone therapy and immunotherapy can kill both cancer and normal cells (Cooper, 1993). Hence the potential usage of natural products as anticancer treatment has been explored intensively by the scientists (Wan-Nor Izzah *et al*., 2009).

CT is originally related to the Leguminosae (Fabaceae) family (Morris, 2009). The vines of this plant are climbing, herbaceous, tall and slender with five leaflets, while the flower colour ranges from white to blue with a white or yellowish center (Jain *et al*., 2003). It is known as butterfly pea and commonly known as Bunga telang in Malaysia. Flowers of CT are consumed to make Nasi Kerabu blue in colour, which is a famous local dish in Malaysia. All parts of CT are useful for medical treatments and have been used in folk medicines and for curing different diseases (Mukherjee *et al.*, 2008). The primary objectives of this study were to determine the chemical composition of the CT's flowers and examine the effect of its aqueous and methanolic extracts on normal and cancer cell lines.

## Materials and Method

Flowers of CT, harvested in October 2010

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were obtained from Kampung Seronok, Bayan Lepas, Pulau Pinang, Malaysia. Hs27 (ATCC® CRL-1634™, human foreskin fibroblast cell line), MCF-7 (ATCC® HTB-22™, hormone-dependent breast cancer cell line), MDA-MB-231 (ATCC® HTB-26™, non-hormone-dependent breast cancer cell line) Caov-3(ATCC® HTB-75™, human ovary cancer cell line), Hela (ATCC® CCL-2TM, human cervical cancer cell line) and HepG2(ATCC® HB-8065™, human liver cancer cell line) were purchased from the American Type Culture Collection (ATTC), USA. Phosphate Buffer Solution (PBS) tablets were obtained from AMRESCO INC, Cleveland, Ohio, USA. The media used was Dulbecco's Modified Eagle Medium (DMEM with low glucose and high glucose) and Foetal Bovine Serum (FBS), penicillin–streptomycin and trypsin were obtained from Gibco®, InvitrogenTM, USA. MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) labelling reagent was obtained from Molecular Probes®, InvitrogenTM, Oregon, USA.

*Sample preparation*

Flowers of CT were separated from the stem and sun dried at room temperature (20°C ± 5°C) for 4 days. Plant powders were kept in an airtight polyester container at -20°C before use.

*Proximate analysis*

Proximate Analysis was determined by using the method stipulated by Official Agricultural Chemists (AOAC) International (1990). The six analyses are including moisture, ash, fat, protein content, fiber and carbohydrate as well.

*Determination of mineral and heavy metals content by Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES)*

The mineral and heavy metal content determined in the flowers of CT included calcium (Ca), zinc (Zn), iron (Fe), sodium (Na), manganese (Mn), copper (Cu), nickel (Ni), chromium (Cr), lead (Pb), boron (B), calcium (Ca), cobalt (Co), potassium (K), magnesium (Mg), selenium (Se), arsenic (As) and cadmium (Cd) as well. This analysis was performed by using Inductively Coupled Plasma Optical Emission Spectrophotometry (ICP-OES) (OPTIMA 7000DV, Perkin Elmer, USA).

*Digestion and sample preparation*

Powder dry samples were weighted 1 gram (n = 3) in microwave digester tubes and digested in (10:1) mixture of nitric acid 65% ($HNO_3$) and perchloric acid ($HCLO_4$). Samples were made to undergo digestion in mineral digester for 60 minutes. After digestion, a few drops of concentrated hydrochloric acid (HCl) were added. The solution was heated gently and then filtrated. Subsequently, the entire filtrate were transferred into a 100 mL volumetric flask and marked up with de-ionized water. The dilute filtrate solutions was transferred into medicine bottles and then injected into the ICP-OES.

*Extraction of sample for anti-proliferative properties*

Hot water was used for the aqueous extracts and methanol was used for the organic extracts. Extraction was done by soaking the CT flowers in boiling distilled water in the proportion of 1:20 (w/v) for 4 hours. The resulting crude extracts were filtered and lyophilized (Huang *et al*., 2003).

The methanol extract was obtained by maceration of the powdered flowers in 95% methanol for 24 hours. The methanol fraction was collected and the residual solvent eliminated by reduced pressure at 40°C by using a rotary evaporator. The residue obtained was dried in a desiccator until it reached a constant weight (Wicaksono *et al*., 2009). The extract produced was used to screen the antiproliferative properties and stored at -20°C until use. The extract was diluted in PBS and then sterilized before assays. Final serial dilution was contained in DMEM with 20% FBS.

*Cell culture*

MCF-7, MDA-MB-231, Caov3, Hela and HepG2 were grown in DMEM with low glucose, and the Hs27 was grown in DMEM supplemented with additional 4.5 g/L of glucose that was used as a comparison. The cells were cultured in the growth medium (supplemented with 10% FBS and 1% penicillin-streptomycin) and incubated overnight at 37°C with 5% $CO_2$ using 25 $cm^2$ tissue culture flasks (McAteer and Douglas, 1979).

*Cell subculture*

The cells were grown to 70-80% confluency by the method of Freshney (1994). Firstly, the old medium was removed, and subsequently the cells were rinsed with PBS twice to wash the cells. The subconfluent monolayer was trypsinized and incubated at 37°C and 5% $CO_2$ for 5 minutes. About 1-2 ml of medium was added into the flask and the cells were collected in growth medium containing serum. The cells were then re-suspended in growth medium, and counted. The total number of viable cells was counted by a haemocytometer to prepare the cell suspension. Sixty microlitres of suspension containing 3 x $10^3$ cells/ ml was added to each well of a 96-well microtiter plate. The plate was then incubated overnight at 37°C with 5% $CO_2$.

*Measurement of the growth inhibitory effect*

Each of the cancer cell lines was grown in a 96-well microtiter plate (Nunc, Denmark) in a volume of 60 µL culture medium per well. The normal and cancer cells were then treated with 60 µL extracted of CT flowers, which contained a serial dilution at doses of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL and the temperature was maintained at 37°C with $CO_2$ for 24–72 hours. The cells in the first row of the 96-well microtiter plate were feed with fresh growth medium for control. After the incubation period, 24 µL of MTT - formazan labelling reagent was added to each well. The microtiter plate was then incubated again for 4 hours at 37°C with 5% $CO_2$. At the end of the drug period, the medium and MTT were removed from all of the wells. Subsequently, the remaining MTT- formazan crystals were solubilised with 100 µL of acidified-isopropanol. One hundred microlitre distilled water was added into each well for further colour development. The absorbance of viable cells was measured using a spectrophotometric plate reader (Multiskan spectrum, Thermo Electron Co., Waltham, Massachusetts, USA) at 570 nm immediately, due to unstable product. To calculate the $IC_{50}$, the processes giving below were followed.

$$\text{Cell viability (\%)} = \frac{\text{OD of drug- tested sample - OD of Blank}}{\text{OD of Control – OD of Blank}} \times 100$$

Dose response curve were constructed using probit analysis (Finney, 1962) on a finney computer program Bio Stat™ 2009 (AnalystSoft Inc., Vancouver, Canada) to obtain $IC_{50}$ value.

*Statistical analysis*

Results for percentage cell viability were reported as means ± standard error of triplicate measurements. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Duncan test with $\alpha = 0.05$ using the SPSS statistical package (ver.19.0).

## Results and Discussion

Results of the proximate analysis, mineral and heavy metal content of the CT flowers are presented in Tables 1. In proximate analysis, the parameters determined were moisture content, ash, crude fat, crude protein and crude fiber as well. The flowers of CT contained appreciable amount of crude fiber (2.1 ± 0.2) and fat (2.5 ± 0.1). A dietary pattern containing low-fat and high-fiber products has been associated with reduced risks of breast cancer (Kushi *et al.*, 2012; Rabeta *et al*., 2009). Results also indicate that the flowers of CT are rich in calcium (3.09 mg/g) and magnesium (2.23 mg/g). The potassium, zinc, sodium and iron concentrations of the flowers on this plant were clearly high (1.25, 0.59, 0.14 and 0.14 mg/g), respectively ($p < 0.05$) than most of the other parameters analyzed (< 0.01 mg/g). In addition, minerals such as calcium and magnesium are necessary for growth, skeletal development and other vital processes within the body. Iron is useful for the prevention of anemia and other related diseases (Oluyemi *et al.*, 2006) while zinc plays a role in protein synthesis, normal body development and recovery from illness (Muhammad *et al*., 2011). Deficiency of these nutrients and minerals can be detrimental to human health. The heavy metals tested were at very low levels thus making the plants relatively safe for consumption.

**Table 1.** Proximate analysis, mineral and heavy metal content of flowers of CT

| Proximate | | Mineral | | Mineral | | Heavy metal | |
|---|---|---|---|---|---|---|---|
| **Moisture** | 92.4±0.1 | **Boron** | 0.0150±0.002 | **Magnesium** | 2.2306±0.134 | **Cadmium** | <0.0001 |
| **Ash** | 0.45±0.15 | **Calcium** | 3.0953±0.09 | **Manganese** | 0.0249±0.003 | **Arsenic** | <0.0001 |
| **Fat** | 2.5±0.1 | **Cobalt** | <0.0001 | **Molybdenum** | $0.0001 \pm 10^{-4} \times 5.7$ | **Lead** | 0.002333±0.0002 |
| **Protein** | 0.32±0.03 | **Chromium** | 0.0007±0.0 | **Sodium** | 0.1413±0.003 | **Nickel** | 0.001267±0.0001 |
| **Crude Fibre** | 2.1±0.2 | **Cupper** | 0.0103±0.0004 | **Selenium** | <0.0001 | | |
| **Carbohydrate** | 2.23±0.3 | **Iron** | 0.1441±0.007 | **Zinc** | 0.5980±0.006 | | |
| | | **Potassium** | 1.2506±0.235 | | | | |

Data are mean values ± standard deviation (SD) of triplicate results; for proximate analysis, dry basis and are expressed in percentage (%) and results for mineral and heavy metal (mg/g).

*Gas Chromatogram- Mass Spectrometry analysis*

GC-MS analyses were conducted to analyze volatile compounds by using a GC system coupled to a mass selective detector. The column was VB-1 (30 m x 320 µm). The temperature programming for the operating condition was: initial oven temperature, 50°C for 0 min increased up to 260°C at a rate of 10 °C/min and held for 9 min; Injector temperature, 260°C; split ratio, 100:1; carrier gas, helium, solvent delay for 1.70 min; transfer temperature, 260°C; ion source temperature, 260°C and mass range 28 to 400 Da.

Figure 1 shows the percentage inhibition exerted by the water extracted on normal cell line (Hs27) and various human cancer-origin cell lines such as, MCF-7, MDA-MB-231, Caov-3, Hela, and HepG2. Based on The inhibition of cancer-origin cell lines increased steadily with increasing concentrations of the water extract and time duration. Growth

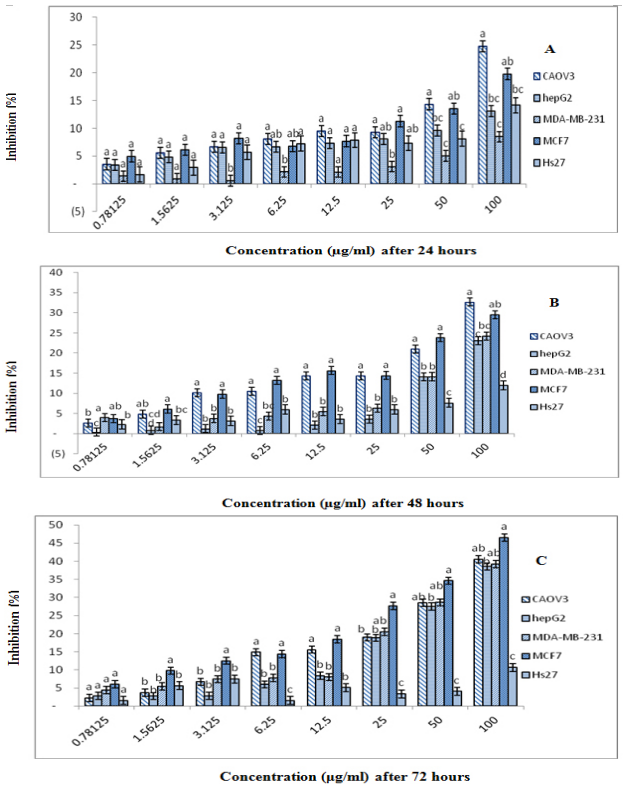



**Figure 1.** Inhibition of water extract of flowers from CT on MCF-7, MDA-MB-231, Caov3, Hela, HepG2 and Hs27 respectively. A) Treated in 24 hours. B) Treated in 48 hours. C) Treated in 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements. (a, b, c) Same letters in each concentration are not statistically significant from each other at $p < 0.05$

inhibition of the cancer origin cell line was most significant ($p < 0.05$) at 72 hours. The water extract had no effect on normal (non cancer-origin) cell growth. After treatment with the water extracted, the inhibition showed more anti proliferations for MCF7, Caov3, HepG2 and MDA-MB-231 with $IC_{50}$ values of 175.3 µg/mL, 224.5 µg/mL, 236.3 µg/mL, and 304.7 µg/mL at 72 hours, respectively but the growth of Hela wasn't effected with the water extract (Table 2). Figure 2 emphasizes the growth inhibitory effect of the methanol extract on MCF-7 cell lines ($IC_{50}$= 536.01 µg /mL), and MDA-MB-231 cell lines ($IC_{50}$= 561.3 µg/mL) at the 72 hours mark ($p < 0.05$). In contrast, the methanol extracted had no effect on the growth of Caov-3, Hela and HepG2 (Table 2). The water extracted of CT was found to have a stronger antiproliferative effect in comparison with the methanolic extract ($p < 0.05$). This may be due to the presence of more active compounds into the water extract.

Several studies have shown that the cytotoxicity and anticancer properties of natural plant are mainly due to the presence of flavonoids. Phenolic compounds, including flavonoids are especially promising candidates for cancer prevention. This could have

**Table 2.** The $IC_{50}$ of water and methanol extract from flowers of CT on cancer cell lines

| Cell lines | 24 hr | | 48 hr | | 72 hr | |
|---|---|---|---|---|---|---|
| | Water | Methanol | Water | Methanol | Water | Methanol |
| Caov3 | 8386.5 | 41333.1 | 857.1 | 2109.9 | 224.5 | 947.2 |
| Hela | 42274.3 | 20381.7 | 18835.4 | 6281.1 | 51513.7 | 2095.7 |
| HepG2 | 1438512 | 40674.6 | 481.5 | 23880 | 236.3 | 5214.1 |
| MDA-MB-231 | 55355.3 | - | 481.5 | 4343.6 | 304.7 | 536.01 |
| MCF-7 | 42567.6 | - | 1159.2 | 1983.4 | 175.35 | 561.3 |

Using Probit analysis on a finney computer program Bio Stat™ 2009.

Probit analysis is a type of regression used to analyze binomial response variables.

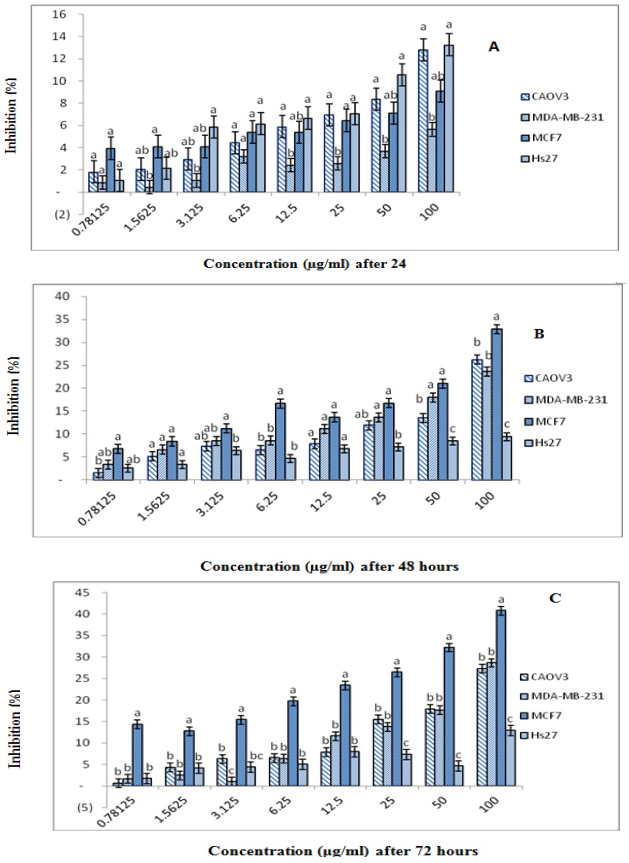



**Figure 2.** Inhibition of methanol extract of flowers from CT on MCF-7, MDA-MB-231, Caov3, Hela, HepG2 and Hs27 respectively. A) Treated in 24 hours. B) Treated in 48 hours. C) Treated in 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements. (a, b, c) Same letters in each concentration are not statistically significant from each other at $p < 0.05$

contributed to the susceptibility of the cells to be aqueous extract of CT flowers. Furthermore, Rajan *et al.*, (2011) reported similar result that showed extracts of petroleum ether from CT had cytotoxic activity against HepG2 cell line. Shyam Kumar and Bhat (2011) also added that petroleum ether extracted and ethanolic extract of CT flowers had cytotoxic activity against the Dalton's Lymphoma Ascites (DLA) cell

**Table 3.** Potocomponents identified in the water extract CT flowers by GC-MS

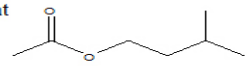
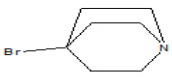
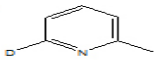
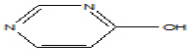
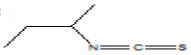
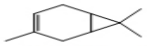
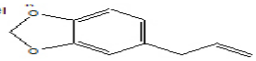
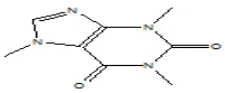
| RT | Chemical compound and structure | Area% | R.Match | Prob | formula | Molecular Weight |
|---|---|---|---|---|---|---|
| 2.187 | Pentanal  | 14.384 | 745 | 21 | C5H10O | 86.13 |
| 3.003 | l-gala-l-ido-octose | 3.509 | 559 | 15.3 | C7H14O2 | 130.18 |
| 6.138 | 1-butanol, 3-methyl-, acetat  | 3.189 | 803 | 20.2 | C7H14O2 | 130.18 |
| 13.534 | mome inositol | 38.7 | 864 | 83.7 | C7H14O6 | 194 |
| 14.219 | 4-bromoquinuclidine  | 1.434 | 754 | 23.1 | C7H12BrN | 190.08 |

**Table 4 .** Potocomponents identified in the methanol extract from flowers of CT by GC-MS

| RT | Chemical compound and structure | Area% | R.Match | Prob | formula | Molecular Weight |
|---|---|---|---|---|---|---|
| 2.019 | acetic acid, cyano-  | 6.523 | 899 | 29.7 | C3H3NO2 | 85.06 |
| 2.49 | pyridine-2-d, 6-methyl-  | 1.643 | 759 | 67.9 | C6H6DN | 94.13 |
| 2.737 | Hirsutene | 5.727 | 851 | 67.7 | - | - |
| 3.225 | pyrimidine, 4-hydroxy-  | 0.919 | 707 | 16.3 | C4H4N2O | 96.09 |
| 3.443 | butane, 2-isothiocyanat  | 0.663 | 541 | 12.7 | C5H9NS | 115.2 |
| 4.609 | bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-  | 0.464 | 895 | 11.6 | C10H16 | 136.23 |
| 5.687 | cyclohexen, 1-methyl-4-(1-methylethylideme)-  | 7.14 | 924 | 17.7 | C10H16 | 136.23 |
| 8.607 | 1,3-benzodioxole, 5-(2-propei  | 1.782 | 955 | 64.7 | C10H10O2 | 162.19 |
| 12.638 | benzoic acid, 2,4-bis(trimethylsiloxy)-trimethylsilyl ester | 0.586 | 737 | 18.6 | - | - |
| 12.913 | mome inositol | 0.792 | 612 | 11.6 | C7H14O6 | 194 |
| 13.133 | mome inositol | 3.952 | 759 | 51.5 | C7H14O6 | 194 |
| 13.286 | 1-nitro-2-acetamido-1,2-dideoxy-d-monnitol | 0.879 | 543 | 21.4 | - | - |
| 13.709 | mome inositol | 33.618 | 877 | 87.1 | C7H14O6 | 194 |
| 15.23 | Caffeine  | 1.943 | 936 | 74.1 | C8H10N4O2 | 194.19 |
| 16.333 | hexadecanoic acid  | 0.529 | 884 | 46.2 | C16H32O2 | 256.42 |

line. Based on the growth inhibitory properties of the extracts observed, GC-MS analysis were obtained.

The components presented in the water extract from flowers of CT were mome inositol (38.7%) and pentanal (14.3%) (Table 3). The GC-MS chromatogram of the methanol extracte (Table 4) showed 15 peaks indicating the presence of three phytochemical constituents. The components presented in the methanol extract from flowers of CT were mome inositol (33.6%), cyclohexen, 1-methyl-4-(1-methylethylideme)- (7.1%), acetic acid, cyano- (6.5%) and hirsutene (5.7%). Furthermore, Studies from *in vitro* experiments, animal studies, and limited clinical experiences, claim that inositol may be used effectively against some types of cancer, particularly when used in combination with phytic acid (Vucenik and Shamsuddin, 2003). These compounds may be responsible for the anti-cancer activity observed during in this study. However, further in vivo study is needed to confirm our findings and evaluating actual anti-proliferative properties in the CT flowers.

## Conclusion

Conclusively, better inhibitions of cancer cell lines were observed in the water extract ($IC_{50}$ of 175.3 µg/ml for MCF7). Knowing the exact compounds responsible for the plant's anticancer properties will

help in formulating anticancer agents. In addition, it results from the proximate and mineral constituent analysis at the plant has provided pertinent information for food formulations.

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