Evaluation of Total Phenolic, Total Flavonoid, and In Vitro Cytotoxic Activity of Syzygium cumini Extract in Cervical Cancer Cell

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Abstract. Side effect of conventional cancer therapy has driven researches to find alternative therapy. People in the province of West Nusa Tenggara, especially the Sasak tribe, perform medical treatments that refer to the traditional Lontar Usada manuscript. One of the plants mentioned in this manuscript was jamblang plant (Syzygium cumini). This study aims to explore and determine the potential of S. cumini leaves extract as an anti-cervical cancer. Extraction was carried out by the Soxhlet method using ethyl acetate, methanol, and water as solvents. The extract obtained was tested for Thin Layer Chromatography (TLC), FTIR test, and cytotoxicity test for the MTT method using HeLa cells. TLC exhibited that the extract contain phenol and flavonoid. FTIR analyzed that the extract had functional groups O-H phenols, C-H alkanes, C=C alkenes, C=O ketone, C=C aromatic ring, NO₂ nitro compound, and C=C alkenes. Total phenol content and total flavonoid content of ethyl acetate, methanolic and water extract were 443.80 ± 0.33; 305.80 ± 0.28; and 45.80 ± 0.11 mg GAE / g, and 74 ± 0.12; 70 ± 0.28; and 34 ± 0.21 mg QE / g. Ethyl acetate extract showed highest cytotoxicity with IC₅₀ value 330.50 ± 1.59, followed by methanol extract 378.35 ± 2.84 and water extract 360.84 ± 0.85.

Keywords: cytotoxic, Syzygium cumini, HeLa

1. Introduction
Cervical cancer is a cancer that occurs in female reproductive system called cervix. This disease is caused by infection of Human Papilloma Virus (HPV) on the surface of cervix which can causing abnormal growth of cervical cells [1]. Cervical cancer ranked 4th in the world with 7.5% mortality rate. In Indonesia itself, estimated case of cervical cancer case is 17 per 100,000 females and increasing every year [2].

The treatment of cancer usually done by chemotherapy, radiotherapy and surgery. However, these conventional therapy did not achieve optimum result and tend to cause side effects which can endanger the patient [3]. The failure of cancer treatment, especially chemotherapy is caused by the low selectivity of anticancer drugs against normal cells, and the resistance of cancer cells to chemotherapeutic agent. The
resistance often leads to reoccurrence of the disease. Besides, these chemotherapy agent has been reported to cause nephrotoxicity and cardiotoxicity [4].

Alternative treatment using herbal medicine believed has minimum side effects than synthetic anticancer drugs [5]. One of the potential medicinal herbal is Syzygium cumini or Jamblang. This plant is used traditionally as medicine by Sasak tribe, communities in the province of West Nusa Tenggara, Indonesia. The usage as medicines referred to the ancient manuscript for healthcare guidelines called Usada. In the manuscript, there are 324 types of diseases using 11 types of animals and 163 types of plants treatments guides. One of the plants mentioned Java plum (Syzygium cumini). The parts of the jamblang plant (leaves, fruit, seeds) are recognized by the surrounding community as a cure for diseases such as stomach pain and dysentery [6].

Previous studies also found that extracts of S. cumini fruits contained phenol compounds such as Kaempferol 7-O-methylether and γ-Sitosterol which had an effect on antioxidant and anticancer activity on AML cell lines in tests for leukemia [7]. In addition, other studies have also found that there is a significant decrease in tumors induced in the stomachs of mice using benzo-a-pyrene compounds [8]. Another research [9] also showed that extract of jamblang seeds can inhibit the growth of breast cancer cell line (MCF-7). Since S. cumini has novel bioactive mixes and promising pharmacological applications, this study aims to discover the capability of S. cumini leaves extract against cervical cancer cell line-HeLa.

2. Materials and methods

2.1. Sample preparation

Jamblang leaf samples were obtained from Malang Regency (485-560 masl) and Pasuruan Regency (500-600 masl). The selected leaves had a fresh condition, with an average size of 14 cm. The obtained leaves were washed, cleaned, and enumerated. A total of 50 grams of leaves were wrapped in filter paper and put in a thimble. Sequential extraction based on [10] with slight modification, was carried out for 4 hours with ethyl acetate, methanol and water solvents. The leaves extracted by ethyl acetate solvent were reused for extraction with methanol as a solvent. After that, the leaves of the methanol extracted solvent were reused for extraction with a water solvent. In order to obtain extracts of ethyl acetate extract, methanol extract, and water extract, each extract was then evaporated and dried.

2.2 Thin Layer Chromatography (TLC) Analysis

Qualitative TLC testing based on [11] and [12] was performed using several eluents with different levels of polarity to obtain solvents that were able to provide good separation and good dye stains. The spots on the TLC plate were monitored under UV light 365 nm and the Rf value was calculated.

2.3 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR analysis was carried out based on [13]. Extracts were taken 1 mg, then mixed with KBr and crushed until homogeneous. The mixture was put into a pellet maker with a pressure of 74 atm and within 5 minutes to obtain a pellet with a thickness of ± 1 mm. The plate was placed on a plate container then measured its absorption with FTIR.

2.4 Total phenolic content (TPC) and total flavonoid content (TFC) analysis

Total phenolic content were measured by the Folin-Ciocalteu's reagent method [14]. A total of 10 mg of the extract were dissolved in 10 mL of methanol or at a concentration of 1 mg / mL. The extract solution (stock solution) was taken as much as 0.5 and added 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water and added with 2.5 ml of 7.5% NaHCO₃. The solution was then allowed to stand for 45 minutes at room temperature. The experiment was repeated three times. The absorbance measurement was carried out at a wavelength of 765 nm. The same procedure was carried out to create a standard curve of gallic acid. Based on absorbance measurements, the total phenol can be read from a standard curve, then the total phenol extract was shown in Gallic Acid Equivalent (GAE) per mg extract.
The total flavonoid levels were measured using the aluminium chloride method [14]. A total of 0.5 mL of stock extract solution was added with 1 mL of 2% AlCl₃. The solution was incubated 60 minutes at room temperature. The experiment was conducted in triplicates. The absorbance measurement was carried out at a wavelength of 415nm. The same procedure was carried out to create a standard quercetin curve. Based on absorbance measurements, the total phenol can be read from a standard curve, then the total phenol flavonoids were shown in quercetin equivalent (QE) per mg extract.

2.5 MTT Assay
HeLa cell line were cultured in DMEM medium supplemented with 10% FBS (Fetal Bovine Serum), 2% penicillin, 2% streptomycin, and 0.5% fungizone. Cells were incubated with 5% CO₂ at 37°C. After the cell’s confluency reached 80%, cells were seeded in 96 well plate an incubate 24 hours at 37°C. Cells that has been harvested were seeded into a 96 well plate with 1x10⁴ cell concentration/well with extracts at various concentration (31.25, 62.25, 125, 250, 500 and 1000 µg / mL) for 24 hours. Same procedure also done for cisplatin as positive control. Add 0.05% MTT in seeded cells in 5% CO₂ at 37°C for 6 hours. The reaction was stopped with addition of 20% (w/v) SDS in 20 mM HCl, incubated for 24 hours, and read with ELISA reader with optical density 595 nm [15].

2.6 Statistical analysis
The experimental design of the study was conducted by completely randomized design (CRD) with two factors, solvents (ethyl acetate, methanol, and water), and variance of concentrations. Each treatment was carried out in three replicates. The result was presented as mean values ± standard deviation. Statistical analysis was performed with IBM SPSS Statistics software (version 25 for Windows). One-way analysis of variance (ANOVA) followed by Tukey’s Honest Significant Difference test was used to identify significant differences between extraction conditions, Probit, and Pearson correlation with 5% significance level. The graphics were made using Microsoft Excel 365.

3. Results and Discussion

3.1 TLC analysis
The results of the TLC test can be seen in Table 1. The ethyl acetate extract of S. cumini leaves had a retention factor (Rf) for the detection of flavonoid and phenolic compounds respectively 0.36 and 0.79; in the methanol extract 0.37 and 0.14; and at water extract 0.36 and 0.11. Observation of silica plate under UV 365 nm showed the formation of fluorescence which were flavonoid (amentoflavone) and phenolic (gallic acid) content [11, 12].

| Extract   | Compound                  | Rf   | Fluorescence | Result |
|-----------|---------------------------|------|--------------|--------|
| Ethyl acetate | Flavonoid (amentoflavone) | 0.36 | Observed     | (+)    |
|           | Phenolic (gallic acid)    | 0.79 | Observed     | (+)    |
| Methanol  | Flavonoid (amentoflavone) | 0.37 | Observed     | (+)    |
|           | Phenolic (gallic acid)    | 0.14 | Observed     | (+)    |
| Water     | Flavonoid (amentoflavone) | 0.36 | Observed     | (+)    |
|           | Phenolic (gallic acid)    | 0.11 | Observed     | (+)    |

Based on the Table 1, it can be seen that each extract contains flavonoid and phenolic compounds. The Rf value indicates the polarity of the phytochemical compounds in the extract and the suitability of the solvent used to facilitate further separation of certain compounds [16]. Solubility of polyphenols is significantly affected by the polarity of the solvent at the time of extraction [17]. According to [18], S. cumini leaves are also rich of quercetin, myricetin, myricitin, flavonol glycosides, 3-O-4-acetyl-L-rhamnopyranoside, esterase, triterpenoids, tannin, and galloyl carboxylase [19].
3.2 FTIR analysis

The results of the FTIR Spectroscopy test can be seen in Table 2. FTIR test showed the types of bonds and functional groups found in the sample based on the peak transmittance (%) versus wavelength (cm\(^{-1}\)). The peak of ethyl acetate, methanol and water shows graphical form like the letter "U" with a strong transmittance pattern (strong, broad) at 3600 to 3200 cm\(^{-1}\) wavenumber showing OH bonds (characteristic of polyphenol compounds) [13]. At frequencies 2970 to 2850 cm\(^{-1}\), strong intensities in the ethyl acetate and methanol extracts were detected as alkanes (C-H) [20].

| Peak (cm\(^{-1}\)) | Bond type | Functional Group |
|-------------------|-----------|------------------|
| Ethyl acetate     | 3415.70   | O-H              | Phenol       |
| 2921.96           | 2925.81   | C-H              | Alkane       |
| 1691.46           | 1699.17   | C=C              | Alkene       |
| Methanol          | 1618.17   | C=O              | Ketone       |
| 1542.95           | 1548.73   | C=C              | Aromatic ring|
| Water             | 1373.22   | NO\(_2\)         | Nitro compound|
| 821.62            | 819.69    | C=C              | Alkene       |

The frequency between 1700 to 1600 cm\(^{-1}\) moderate intensity found in the ethyl acetate and methanol extract can be detected as a functional group from alkenes (C = C). The frequency between 1900 to1500 cm\(^{-1}\) strong intensity found in the extract of methanol and water can be detected as a functional group of ketones (C = O). An area between 1700 to1500 cm\(^{-1}\) of moderate intensity found in the methanol and water extracts can be detected as an aromatic ring (C = C). In the range between 1400 to1300 cm\(^{-1}\) moderate-high intensity can be detected the presence of nitro compounds (NO2) groups. The frequency area of 1000 to 650 cm\(^{-1}\) weak intensity is a detection of the functional group of alkenes (C = C) [13].

3.3 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) Analysis

The phenolic content in each extract was measured by gallic acid equivalent (GAE), while the flavonoid content was measured by quercetin equivalent (QE). The result obtained that the total phenolic and flavonoids from the ethyl acetate, methanol and water extract were 443.80 ± 0.33; 305.80 ± 0.28; and 45.80 ± 0.11 mg GAE / g, and 74 ± 0.12; 70 ± 0.28; and 34 ± 0.21 mg QE / g. The ethyl acetate extract had the highest total phenol and flavonoid content compared to the methanol and water extracts (Figure 1).

![Figure 1](image_url)  
**Figure 1.** Total phenolic content and total flavonoid content of extract *S. cumini* leaves.

Phenolic acids and flavonoid are secondary metabolites that play a considerable role as antioxidant agent, because of their ability to scavenging free radicals [21]. Substitution of hydroxyl groups on phenolic aromatic ring affected their scavenging activity because of their hydrogen donor ability [22]. Meanwhile in flavonoid, their antioxidant and scavenging activities is depend on the position of

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**Table 2. Peak and Functional Group of *S. cumini* leaves extract in FTIR spectroscopy**

| Peak (cm\(^{-1}\)) | Bond type | Functional Group |
|-------------------|-----------|------------------|
| Ethyl acetate     | 3415.70   | O-H              | Phenol       |
| 2921.96           | 2925.81   | C-H              | Alkane       |
| 1691.46           | 1699.17   | C=C              | Alkene       |
| Methanol          | 1618.17   | C=O              | Ketone       |
| 1542.95           | 1548.73   | C=C              | Aromatic ring|
| Water             | 1373.22   | NO\(_2\)         | Nitro compound|
| 821.62            | 819.69    | C=C              | Alkene       |
hydroxyl group and other features in the chemical structure. It has been reported that phenolics and flavonoids is proved to be more effective that carotenoid, vitamin C and Vitamin E as great antioxidant [21].

In a study about the lime plant (Hemigraphis repanda) which is used as a breast cancer drug by the people of North Sulawesi, it was found that the plants had TPC and TFC respectively 33.713 mg GAE / L and 26.933 mg QE / L in ethanol solvents [23]. Exploration [24] in the 9 plants and 15 tinctures most commonly used in traditional Romanian medicine, it is known that the total phenolic and flavonoid in these plants has a range of 5-48 mg GAE / 100 g and 1.3-38 mg QE / 100 g. Based on this comparison jamblang leaf extract has the potential to be a new source of phenolic and flavonoids both for alternative medicine and fortification in food ingredients.

3.4 Cytotoxicity analysis

In the MTT cytotoxic test, results obtained that the 50% inhibition concentration (IC50) of the ethyl acetate, methanol, water and cisplatin (standard) respectively were 330.50 ± 1.59 (moderate toxic); 378.35 ± 2.84 (moderate toxic); 3608.84 ± 0.85 (non-toxic) and 8.11 ± 2.55 (very toxic) μg / mL. Based on these values it can be seen that the IC50 value of the ethyl acetate extract has the smallest value. The smaller the IC50 value, the higher the cytotoxicity of an ingredient. S. cumini had cytotoxicity on HeLa cell with the induction of apoptosis (Figure 2).

![Figure 2. Microscopic performance of HeLa cells against S. cumini leaves extract. (A) untreated cells; (B) 500 ppm ethyl acetate extract; (C) 500 ppm methanol extract; (D) 500 ppm water extract.](image)

Plants constitute a common alternative for cancer treatment in the world. They are chosen because of the less toxic to normal cells, more biologically friendly, and consequently more co-evolved with their target sites [25]. In Indonesia, there has been many research and discovery of potential medicinal plants as anticancer. A research using fenugreek seeds with ethyl acetate solvents had low IC50 values obtained in cell lines include 41.81 (MCF7), 58.63 (T47D) 66.24 (PC3) and 208.74 (SKOV3) μg / mL [26]. Study of herb alfalfa (Medicago sativa L.) also found that its ethanol extract had IC50 values in T47D and HeLa cells respectively 523.9 and 503.5 μg / ml [27]. Study by [28] using A. comosus with methanol as extract resulting IC50 741.46 μg / ml in T47D cells. Then ethanol extracts from avocado leaves had IC50 values of 360 μg / ml in HeLa cells [29]. Based on the comparison it can be seen that there are so many plants that can be use as alternative cancer drug.

However, according to U.S National Cancer Institute (NCI) to be used as anticancer it should have IC50 value less than 20 μg / ml for crude extract and less than 4 μg / mL for pure compounds [30]. When compared to the cytotoxicity test of S. cumini leaves extract which have high IC50, it can be concluded that the extract in this study had weak potential as anti-cancer agent in vitro against HeLa cells. The low ability can be caused by several factors, such as the phytochemical content in S. cumini leaves, the extraction methods, and the antioxidant activity. Study by [9] using S. cumini seed extract with Soxhlet extraction method were able to inhibit 68.52 % of MCF-7 cells at concentration 125 μg / mL. Another explorative research using fruit of C. melo [31] showing 38.38% antioxidant activity and able to inhibit 53.55% cancer cells at concentration 100 μg / mL. Study by [7] using S. cumini fruit and extraction with successive solvent resulting 39.9% anticancer activity and 71.07% antioxidant activity
at 100 µg / mL with ether as solvent. However, based on the result in this study it can be seen that that *S. cumini* leaves extracts have shown promising activity as chemo-preventive agent.

### 4. Conclusion

Based on the results, it can be concluded that *S. cumini* leaves extract is categorize as moderate toxic in cytotoxicity test and had weak potential to be used as anticancer agent against HeLa cells. But it has promising activity to be used as chemo-preventive agent.

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