Identification of Anti-cancer Compounds and Cytotoxic Effects on HeLa Cervical Cancer Cells from *Cyperus rotundus L*. Growing in Tanggamus, Lampung, Indonesia.

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Abstract. This study aims to identify the anti-cancer compounds found in *Cyperus rotundus L*. tubers originating from Tanggamus, Lampung, Indonesia and then perform a cytotoxic test on Hela cervical cancer cells. The identification of compounds will be carried out using the GC-MS method, while the cytotoxic test will be carried out using the MTT Assay method. There are 142 compounds were detected with thirteen highest compounds, namely *d-Selinene* (33,02%); *Naphthalenone* (11,49%); *Longiverbenone* (2,91%); *Caryophyllene oxide* (2,78%); *1-Phenanthrenol* (2,35%); *1.4,6-Trimethil-1,2,3,3a,4,7,8,8a-octahydro-4,7-ethanoazulene* (2,28%); *α-Copaene* (2,11%); *Ylangenal* (1,66%); *Panaxjapyne A* (1,31%); *Guaia-1(10),11-diene* (1,30%); *Ledene oxide-(II)* (1,16%); *Isospathulenol* (1,15%); *Methyl-10,12-pentacosadiynoate* (1,14%). Various components identified from GC-MS analysis including terpenoids (sesquiterpene hydrocarbons, oxygenated sesquiterpenes and monoterpenes) which are the anticancer bioactive compounds. Cytotoxic test showed that *Cyperus rotundus L.* tuber extract had a moderate cytotoxic effect (IC_{50} 95,36 µg / ml) on HeLa cervical cancer cells.

1. Introduction

Cervical cancer is one of the most common cancers which can causes infertility, morbidity, and mortality of women in the world. Based on Global Cancer Observatory (GCO) 2020, there are 36.633 new cases of cervical cancer in all ages and 21.003 mortality cases of cervical cancer throughout the world [1]. According to World Health Organization (WHO), cervical cancer is the most common cancer in women with an estimated 570,000 new cases in 2018 which represents 6.6% of all women cancers cases [2]. Indonesia has a cancer incidence rate of 136.2 / 100,000 population, which means Indonesia is in 8th place in Southeast Asia, while 23rd in Asia. Based on Basic Health Research, the prevalence of cancer in Indonesia shows an increase from 1.4 per 1000 population in 2013 to 1.79 per 1000 population in 2018 [3]. Then the incidence rate for cervical cancer of 23.4 per 100,000 population with an average death rate of 13.9 per 100,000 population [4]. The high incidence of cancer is due to an increase in cancer-causing factors and problems in its treatment [3].

Cancer is caused by multifactorial causes, one of the causes is free radicals. Free radicals can damage cells and can change DNA or deactivate genes involved in cell proliferation and death. Thus normal cells can develop into cancer cells. Cancer treatment is currently carried out in several ways including surgery, radiotherapy, chemotherapy as well as hormone and biological therapy. However, the use of chemotherapy drugs has a "bad image" in society because the unwanted effects such as hair loss, stress on the bone marrow, drug resistance, damage to the gastrointestinal system, neurological dysfunction, toxicity to the heart and the most important effect is not only can kill the cancer cells, but also the normal cells [5,6]. Another problem is the treatment so expensive [7,8]. World cancer anniversary day 13 February 2018, it was stated that based on the Insurance and Social Security (Healthcare BPJS) Indonesia report, the burden of cancer costs until September 2017 had spent 2.1 trillion rupiah for cancer treatment [9].

Expensive medication and dreaded side effects are a fear in itself for the community so that currently many researchers are exploring natural ingredients with the aim to finding new anticancer substances. The using of natural product is relatively safer because the side effects are relatively small when compared to chemical drugs, chemotherapy, surgery and radiation. Anticancer compounds play an important roles including inducing Reactive Oxygen Species (ROS), mitotic kinase inhibitors, anti-mitosis, angiogenesis inhibitors, topoisomerase inhibitors, cause the apoptosis, and inhibit the cancer proliferation [10]. The proving of bioactive compound can be toxic for cancer cells can be using cytotoxic test and antiproliferative test in vitro [11]. There are many natural ingredients which are good sources in the development of new medicines for various diseases [5,8]. One medicinal plant that has the potential to be developed as an anticancer is *Cyperus rotundus L*. [12].

The use of *Cyperus rotundus L*. as an alternative treatment source has grown rapidly in the past decade. *Cyperus rotundus L*. is one type of perennial weeds that is always around cultivated plants because it is able to adapt well. It belongs to the Cyperaceae family and is widespread in tropical, subtropical and temperate regions [13]. *Cyperus rotundus L*. contains bioactive compounds and high nutritional value. It contains primary metabolites such as carbohydrates, proteins and amino acids, then secondary metabolites such as flavonoids, tannins, glycosides, furochromone, monoterpenes, sesquiterpenes, sitosterols, alkaloid saponins, terpenoids, and essential oils [14]. The essential oils of this plant from different countries also showed differences of compounds. In China the most abundant constituents of the volatile oils are *α-cyperone* (29,38%), *cyperene* (13,97%), *caryophyllene oxide* (6,71%) and *β-selinene* (6,47%), but in Tunisia showed *cyperotundone* (19,7%), *cyperene* (15,2%), *mustakone* (5,8%), *caryophyllene oxide* (2,6%), *rotundene* (3,6%) dan *eudesma 5-en-11-α-ol* (2,6%) [15,16]. The variety of composition and concentration of chemical compounds contained in the essential oil of *Cypers rotundus* plants living in different countries indicate the environmental influence on the chemical qualities of essential oils in plants. Phytochemical research can be used to find chemical compounds for medical treatment. One of the effects of the bioactive compounds in *Cyperus rotundus* L. which has been widely studied is as an anticancer. [17].

2. Materials and Methods

2.1. Materials

The fresh rhizomes of *Cyperus rotundus L*. were collected from district of Tanggamus, Lampung, Indonesia. The latitude and longitude coordinates are: 5°22'38.2"S -104°47'33.2"E. Samples were brought to the Botany Laboratory, Faculty of Mathematics and Sciences, University of Lampung for taxonomic verification.

2.2. Extraction

The tuber of *Cyperus rotundus L*. were washed, drained, then dried in the oven at 50°C for 24 hours. Dry tubers are made into powder using a grinder machine until smooth. The powder weighed ± 200 g. Then the powder was extracted by maceration using chloroform solvent (400 ml), then stirred with a stirrer for 30 minutes and let stand 24 hours, then filtered with a vacuum filter. The filtrate is taken and

evaporated, the residue is added with chloroform, stirred for 30 minutes, and left for 24 hours then filtered with a vacuum filter. The filtrate I, II, and III were mixed and evaporated with a pressure evaporator <760 mmHg. After it becomes thick, it is then poured into a cup, then dried until the chloroform extract is ready for use.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS)

The Analysis of GC-MS compounds was carried out using GC - QP2010 (Shimadzu) equipped with Omegawax TM250 ID fused silica capillary column (30m X 0.25mm, 0.25µm film thickness). The instrument was set to an initial temperature of 100°C and an injection temperature of 270°C with a column flow rate of 1.21 ml/min. The carrier gas is helium with a flow rate of one ml/minute and a linear velocity of 35 cm/sec. All compounds were identified by comparison of mass spectra and known component retention index data found in the literature and spectrum databases kept in the GC-MS library. The data obtained from the analysis of the compound content using the GC-MS method were displayed descriptively using tables

2.4. Cytotoxic Test

The Cytotoxic test of chloroform extract of *Cyperus rotundus L*. against HeLa cells was carried out using MTT assay method, testing each extract using a concentration of 500; 250; 125; 62,5; 31,25; 15,63; 7,81; 3,9 µg/ml. The first step was planting of 100 µL of Hela cells in RPMI medium in microplate 96-wells, some wells were only filled by RPMI medium as a control. Cell culture was incubated for 24 hours in an incubator at 37°C and 5% CO2. After incubation, culture medium in wells were removed. The wells of cell control and medium control were added 100 µL of new RPMI medium, while the wells of treatment were added 100 µL of suspension from RPMI medium and the extract according to each treatment concentration. Cell culture was re-incubated for 24 hours. After the incubation, medium were removed and replaced with a new one, then at each well 10 µL of MTT reagent (3- (4-5 dimethylthiazol2-yl) -2, 5- diphenyl tetrazolium bromide) were added and incubated for 4 hours. Then the 50 µL of reagent stopper (sodium dodecyl sulfate 10%) were added. Microplate was wrapped in aluminum foil, and incubated at room temperature overnight. In the last stage, absorbance of the cell was read using an ELISA reader at a wavelength of 595 nm. Results obtained were used to calculate the percentage of living cells and IC_{50} values for each treatment. The absorbance data obtained from the cytotoxic test was converted into percent of living cells by below formula.

$$\text{Percent of living cells} = \frac{\text{absorbance of treatment} - \text{absorbance of media}}{\text{absorbance of control} - \text{absorbance of media}} \text{ x } 100\% \quad (1)$$

Data on the percentage of living cells was converted to IC_{50} by using the formula that will be converted into a dose-response curve with probit analysis, then the IC_{50} is obtained from each test material so that the conclusion has a cytotoxic effect or not.

3. Results

The compounds that identified in the chloroform extract of *Cyperus rotundus L* using GC-MS are presented in Table 1. Among 142 compounds were detected, there are thirteen highest compounds, namely *d-Selinene* (33,02%); *Naphthalenone* (11,49%); *Longiverbenone* (2,91%); *Caryophyllene oxide* (2,78%); *1-Phenanthrenol* (2,35%); *1.4,6-Trimethil-1,2,3,3a,4,7,8,8a-octahydro-4,7-ethanoazulene* (2,28%); *α-Copaene* (2,11%); *Ylangenal* (1,66%); *Panaxjapyne A* (1,31%); *Guaia-1(10),11-diene* (1,30%); *Ledene oxide-(II)* (1,16%); *Isospathulenol* (1,15%); and *Methyl 10,12-pentacosadiynoate* (1,14%).

Table 1. Thirteen highest compounds that identified in the chloroform extract of *Cyperus rotundus L* using GC-MS

Number	Compound	Retention Time (min)	Peak Area (%)
1	alfa-Copaene	19,45	2,11
2	d-Selinene	20,11	33,02
3	1,4,6-Trimethyl-1,2,3,3a,4,7,8,8a-octahydro-4,7-ethanoazulene	21,60	2,28
4	Guaia-1(10),11-diene	22,44	1,30
5	Isospathulenol	23,35	1,15
6	Caryophyllene oxide	24,65	2,78
7	Ledene oxide-(II)	25,26	1,16
8	Ylangenal	25,52	1,66
9	Longiverbenone	26,85	2,91
10	2(1H) Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)	27,26	11,49
11	Panaxjapyne A	29,62	1,31
12	Methyl 10,12-pentacosadiynoate	31,87	1,14
13	1-Phenanthrenol	37,59	2,35

Referring to previous reports in introduction about the chemical composition of *Cyperus rotundus L.* in China and Tunisia, the results of the current study seem to little different. The differences in the findings of this study with previous research results elsewhere, actually confirm that the chemical content of the plant is influenced by both genetic and environmental factors [18,19]. Overall, the compounds of *Cyperus rotundus L* consisted mainly of phenolics, terpenoids and steroids which are the anticancer bioactive compounds. Certain phenolics are known to possess cytotoxic activity on various cancer cell lines by activation of caspase mediated apoptosis [20]. Phytosterols exert anticancer activity by inhibition of cancer cell proliferation, angiogenesis and induction of apoptosis/necrosis [21]. Terpenoids constitute an important class of phytochemicals with antioxidant and anticancer activities. Certain terpenoids such as D-limonene, perillyl alcohol and salvicine have shown interesting antitumor activity in pre-clinical studies with minimal cytotoxicity on normal cells [22]. The extract has shown a potent antioxidant and free radical scavenging which further may contribute to its anticancer activity [23]. The testing of cytotoxic effects can be observed directly using an inverted microscope. The morphological observations of HeLa cancer cells after the treatment by chloroform extract of *Cyperus rotundus L* and MTT reagent can be seen in Figure 1.

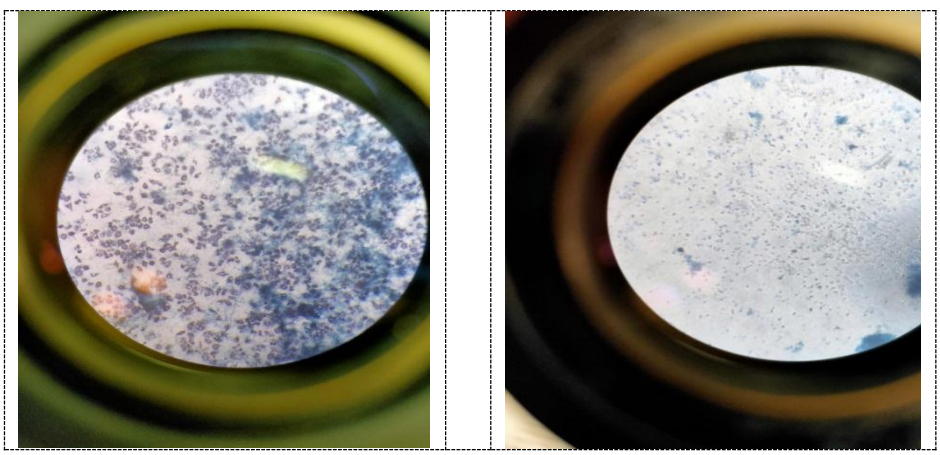

Figure 1. Morphology of HeLa cells in MTT assay test. Cell control (medium + HeLa cells).		**Figure 7.** Morphology of HeLa cells in MTT assay test. HeLa cells after MTT tested (medium + HeLa cells + chloroform extract of *C. rotundus L* 500 µg/ml

Cytotoxic test using the MTT assay method was used to calculate the ability of a bioactive compound to inhibit cancer cell growth determined by IC_{50} value (concentration that can inhibit cell growth by 50%). IC_{50} value was obtained from calculations with probit analysis of the relationship between the percentage of living cells and the concentration of *Cyperus rotundus L*. extracts. The results of probit analysis can be seen in Figure 2.

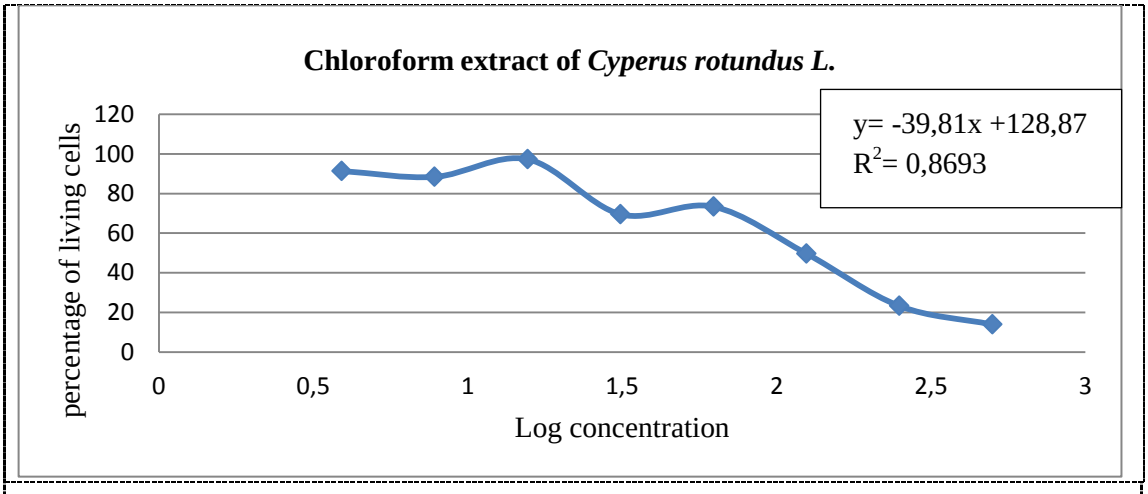

Figure 2. Graph of probit analysis with log concentration of *Cyperus* rotundus L. chloroform extract on the percentage of living HeLa cells..

Table 2. Results of calculating IC_{50} values

Formula	IC_{50} (µg/ml)
y= 50 y= -39,81x +128,87 $IC_{50}= 10^{x}$	95,36 µg/ml

The results of the analysis showed that IC_{50} value chloroform extract of *Cyperus rotundus L.* was 95,36 µg/ml that means it could inhibit the growth of HeLa cells with moderate cytotoxic. Research using the same method has been widely reported. Methanol extract of *Cyperus rotundus L*. flowers has a weak cytotoxic effect on K562 and L1210 leukemia cells through the induction of apoptosis [24]. The ethanol extract and n-hexane fraction of nuts rhizome also have cytotoxic activity on MCF-7 breast cancer cells [25], Erlich carcinoma [26], and oral cancer cells [27].

4. Conclusions

There were 142 components detected with the highest thirteen compounds in *Cyperus rotundus L.* tubers originating from Tanggamus, Lampung, Indonesia. Among all, the thirteen predominant compounds are *d-Selinene*; *Naphthalenone*; *Longiverbenone; Caryophyllene oxide*; *1-Phenanthrenol*; *1.4,6-Trimethil-1,2,3,3a,4,7,8,8a-octahydro-4,7-ethanoazulene*; *α-Copaene*; *Ylangenal*; *Panaxjapyne A*; *Guaia-1(10),11-diene*; *Ledene oxide-(II)*; *Isospathulenol*; and *Methyl 10,12-pentacosadiynoate*.

Various components identified from GC-MS analysis including terpenoids which are the anticancer bioactive compounds. Cytotoxic test showed that *Cyperus rotundus L.* tuber extract had a moderate cytotoxic effect (IC_{50} 95,36 µg / ml) on HeLa cervical cancer cells

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