Jurnal Natural
Vol. 22, (1) 2022
DOI 10.24815/jn.v22i1.22854
pISSN 1411-8513
eISSN 2541-4062
ORIGINAL RESEARCH

Anticancer activity and apoptotic induction of *Chromolaena odorata* Linn leaves extract and fractions on hepatocellular carcinoma cell lines (HepG2)

HANIFAH YUSUF[1*], MARHAMI FAHRIANI[2]

[1]Department of Pharmacology, Faculty of Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia
[2]Medical Research Unit, Faculty of Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia

Abstract. This study evaluates the anticancer and apoptosis activity of *Chromolaena odorata (C. odorata)* leaves extract on HepG2 cancer cell lines. The *C. odorata* leaves were extracted by maceration using ethanol 80% then fractionated by using n-hexane, ethyl acetate, and ethanol. MTT assay was used to evaluate the anticancer activity, and flowcytometry was used to evaluate the apoptosis induction. The phytochemical analysis and characterization of the extract's major compound were done using Liquid Chromatography-Mass Spectroscopy (LCMS). The phytochemical analysis showed that the crude ethanolic extract contained alkaloids, flavonoids, phenolic, steroids, tannin, and saponin. The LCMS showed the major compounds of the extract are 5,7,8,3′,4′-Pentamethoxyflavonone, 1-Carboethoxy-β-carboline, 3-Methylcanthin-2, 6- dion, Canthin-6-one. In the n-hexane fraction, we identified 5,7,8,3′,4′-Pentamethoxyflavonone, Methyl ophiopogonanone A and Pd-C-II. Meanwhile, 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one, 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8- methoxy-chroman-4-one, Methyl opiopogonanone A and Sinensetin was identified in the ethyl acetate fraction. The ethanol fraction contained 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one, 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8- methoxy-chroman-4-one and Methyl opiopogonanone A. The crude ethanolic extract, n-hexane, ethyl acetate and ethanol fractions the IC_{50} value of 23.44 µg/mL, 84.52µg/mL; 88.51µg/mL; and 167.49 µg/mL, respectively. This research suggests that the crude ethanolic extract of *Chromolaena odorata* leaves is a potential candidate for hepatocarcinoma treatment.

Keywords: anticancer activity, cancer cell lines, *Chromolaena odorata* Linn., hepatocarcinoma

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and the spread of the body's abnormal cells[1]. Cancer is the second leading cause of death in high-income countries after heart disease. Meanwhile, cancer is the third highest cause of death in low-income countries after heart disease and infection [2]. Cancer treatment by surgery, radiation, and anticancer (chemotherapy) are quite expensive, especially for low and medium-economic income patients[3]. Several side effects arise from chemotherapy causes people with cancer to stop treatment, and they come to traditional healers to reduce and relieve their suffering[4]. Such conditions have encouraged research on finding alternative medicines derived from natural compounds, especially plants.

Several plants have been used empirically and are believed to have anticancer activity. Some plants have long been known to have anticancer activity, but most have not been scientifically proven[5]. Problems in cancer treatment, such as its side effect, showed that it is crucial to conduct research to find new anticancer compounds hoping that these drugs have better efficacy and safety. To assess the efficacy and safety of a drug, the potency and selectivity can be used as a benchmark[6]. The smaller inhibition value of cancer cell growth shows the better potential of anticancer activity. On the other hand, if the anticancer selectivity index is greater, side effects will be less. Thus, the selectivity of anticancer should be a priority in preventing the adverse effects of anticancer on other parts of the patient's body.

*Corresponding Author:
hans_yusuf1104@unsyiah.ac.id

Received: September 2021| Revised: December 2021 | Accepted: March 2022

Several plants of Eupatorium species, namely *Eupatorium riparium*, are known to have anticancer activity [7], and *Eupatorium adenophorum* Spreng has an IC50 value of 50µg/ml against HepG2 [8]. It is suspected that chemotaxonomically, there is a similarity in chemical content in the same family or genus of these plants. Eupatorium odoratum or *Chromolaena odorata* plants have generally been used from generation to generation to treat various diseases[9]. This plant is included in the Asteraceae family, which contains compounds similar to *Eupatorium riparium*. Another study reported that *Eupatorium riparium* is cytotoxic against hepatocellular carcinoma cell line (HepG2) [10]. Therefore, this study was conducted to determine the anticancer and apoptotic induction of crude ethanolic extract and fractions of *C. odorata* leaf against hepatocellular carcinoma (HepG2) and identify the primary compounds in the extract and the fractions by LCMS.

The crude ethanolic extract of *C. odorata* L was made by the maceration method using 80% ethanol as solvent. Ethanol is a polar solvent which was used to increase the yield of flavonoids in the extracts[11]. Fractination based on solvents with different polarities (n-hexane, ethyl acetate, and ethanol) was performed to extract all the phytochemical and antioxidant compounds present in the plant material[12].

MATERIALS AND METHODS

Collection and identification of plant

The leaves of *C. odorata* Linn were collected from Samahani in Aceh Besar Regency and identified by the expert from the Biology Department, Faculty of Mathematics and Natural Sciences University of Syiah Kuala Banda Aceh with the reference number of B/435/UN11.1.8.4/TA.00.01/2020.

Preparation of plant sample and extract

The collected leaves of *C. odorata* were dried under shade and ground using an electrical grinder into a powder. The dried powder of *C. odorata* leaves (1500 g) was extracted for 72 hours with ethanol 80% (1: 3) in a macerator. The solvent was filtrated every 24 hours then the maceration continued by using the new ethanol 80%. The whole liquid extract was vacuum evaporated until the semisolid extract was obtained. The extract was dried in a desiccator and weighed.

Fractionation of crude ethanolic extracts of *Chromolaena odorata* leaves

The fractionation of crude ethanolic extract of *C. odorata* leaves is done using the liquid-liquid partition method. The concentrated extract of crude ethanolic extract was dissolved in ethanol (7:3), filtered and fractionated with n-hexane in a separating funnel with a ratio of 1:1, and was shaken sufficiently. Then, it was set aside until two layers were formed; the n-hexane and the ethanol layers. This treatment was carried out several times until a pure n-hexane fraction was obtained. The same treatment was repeatedly applied to the ethanol layer using ethyl acetate solvent in the same ratio. The fractionation results in n-hexane, ethyl acetate, and ethanol fractions, then evaporated using a rotary vacuum evaporator to obtain the viscous fractions.

Phytochemical analysis of crude ethanolic extracts and fractions of *C. odorata* Leaves

a. Identification of Alkaloids

50 mg extract was dissolved with chloroform, then 0.5 mL of 1 M sulfuric acid was added. The extracts were shaken slowly and then set aside until two layers formed. The clear layer was divided into half, with 2-3 drops of Dragendorff reagent was added to one part, and 2-3 drops of Mayer's reagent was added to the other half. A brick-red precipitate formed by Dragendorff's reagent and white precipitate by Meyer's reagent indicates the presence of alkaloid compounds.

b. Identification of Flavonoids

2 ml of the aqueous extract and a few drops of Bench concentrated ammonia (NH_4) were added into a test tube. The formation of a yellow color indicated the presence of flavonoids. A confirmatory test was carried out by adding a few drops of concentrated hydrochloric (HCl) into the yellow solution, which turned colorless.

c. Identification of Triterpenoids

The presence of terpenoids was determined using the Salkowski test. 2 ml of the extract was mixed in 2 ml of chloroform in a test tube, then 3 ml of concentrated sulphuric acid (H_2SO_4) was carefully added to form a lower layer. A reddish-brown color interface was indicative of the presence of terpenoids.

d. Identification of Phenolic

0.5 grams of the extract was dissolved in 2 mL of 96% ethanol, then 3 drops of 10% $FeCl_3$ solution was added. The formation of a bluish-black precipitate color indicates the presence of phenolic compounds.

e. Identification of Tannin

0.5 grams of the extract was dissolved in 2 mL of 96% ethanol then 5 drops of 10% of lead acetate was added. The formation of white precipitate indicated the presence of tannins.

Afterward, A total of 2 ml of the aqueous extract was put into a test tube and then 2 drops of dilute ferric chloride ($FeCl_3$) were added. If a blue-black or blue-green precipitate was formed, it indicates the presence of tannins.

f. Identification of Saponin

Saponin was detected using the Froth test as follows: 2 ml of aqueous extract was mixed with 6 ml of distilled water in a test tube, then the mixture was shaken well, and the formation of foam indicated the presence of presence saponins. Another test for detecting saponin was the emulsion test that was performed by adding three drops of vegetable oil into a test tube containing aqueous extract, then the mixture is shaken well. The formation of a stable emulsion indicates the presence of saponins.

*g. Identification of Steroid*s

About 2 ml of acetic anhydride (Ac2O) was added to 5 ml of extract of each sample with 2 ml sulphuric acid (H2SO4). A color changed from violet to blue or green indicated the presence of steroid.

Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was used to identify the major compounds in the crude ethanolic extract and its fraction of *C. odorata* leaves. Chromatographic analysis was carried out by reverse phase elution (LC-18 column 250 × 4.6 mm, 5 μm) on Agilent 6500 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF; Agilent Santa Clara, CA, USA) which was detected by using LC/MS system with Agilent 1200 Series Diode Array Detector. The mobile phase consisted of (A) formic acid / WA (0.1%, v/v); (B) acetonitrile + 0.1% formic acid; gradient (in solvent B), flow rate: 0.2ml/min; injection volume 3 L; ESI parameters: both negative and positive ion mode; mass range 50–1200 *m*/*z*; spray voltage 4 kV; gas temperature 325^0C; gas flow 10 L/min; Nebulizer 40 psi and the mass was analyzed by using Agilent technologies Mass-Hunter software.

Hepatocarcinoma cell culture (HepG2 cancer cell lines)

HepG2 cancer cells are a collection of the Laboratory of Parasitology FKK-MK University of Gadjah Mada Yogyakarta. Cells were grown in DMEM (Sigma) liquid culture medium containing 10% fetal bovine serum (FBS) (Sigma), 100 g/ml penicillin-streptomycin (Gibco), and incubated in a 5% CO_2 incubator and at 37^0C.

Ethical approval

The research approved ethical clearance from the Ethics Commission, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia No. 239/KEPK/FK UNLAM/VI/2020.

Anticancer activity test

The anticancer activity test of *C. odorata* extract and fractions was carried out using the MTT colorimetry method. HepG2 cells with a density of 10,000 cells/well were put into a 96 well microplate, then incubated for 24 hours in a 5% CO_2 incubator at 37^0C. After 24 hours, cells were treated with extract and fractions at concentrations of 7.8, 15.63, 31.25, 62.5, 125, 250, and 500 µg/mL, respectively, and incubated for 24 hours in a 5% CO_2 incubator at 37^0C. The cell morphology was observed at the end of each treatment by using an inverted microscope and documented by a Canon camera. Then, 100 µL culture media with 10 µL MTT 5 mg/mL was added into each well, and incubated at 37^0C for 4 hours in a 5% CO_2 incubator. Intact cells will react with MTT to form purple formazan crystals. Crystals were dissolved with a reagent stopper (SDS 10% in 0.01N HCL) and left aside in a dark place overnight. The optical density was then read by using an ELISA reader on a wavelength of 595 nm and converted into a percentage of living cells by using the formula (1).

Cell apoptotic induction test using flowcytometry method

6-well microplate was filled with 500µL HepG2 cell suspension with a density of $5x10^4$, then incubated in a CO_2 incubator at 37^0C. Cells were then filled with 500µL of n-hexane, ethyl acetate, and ethanol fraction with a concentration of IC_{50}. Growth media and HepG2 cells were used as a negative control. At the end of incubation, cells were centrifuged for 3 minutes at 3000 rpm. The supernatant was discarded, PBS was added to the pellet, and resuspended. The suspension was transferred to a microtube and centrifuged, and then the supernatant was discarded, then cells were harvested with trypsin. Cells were transferred to microtubes and then centrifuged for 3 minutes at 3000 rpm. The supernatant was removed and the cells were rinsed with PBS

$$\% \text{ Living cells} = \frac{(\text{Control of normal cells} - \text{Control of media}) - (\text{Treatment cells} - \text{Control of media})}{(\text{Control of normal cells} - \text{Control of media})} \quad \ldots\ldots..(1)$$

and centrifuged again, then PBS was discarded. The cell precipitate in the microtube was fixed with 70% ethanol at -20^{0}C, incubated for 30 minutes at room temperature, and then centrifuged. The precipitate was washed with PBS twice and then added with Propidium Iodide reagent and Annexin V. The microtubes were wrapped in aluminum foil and incubated in a dark room for 15 minutes. The cell suspension was homogenized and then transferred to flowcytometer tube with a nylon filter, then analyzed. The cell morphology were calculated and compared to the control group.

Statistical Analysis

Experiments were repeated at least three times and results are expressed as mean ± SD. The IC_{50} was calculated by using nonlinear regression analysis. Data on apoptosis were analyzed by two-way analysis of variance (ANOVA) followed by Tukey post-test to determine the significant differences between treatments with p-values <0.05 was considered significant. All statistical analysis were performed using Graph Pad Prism v.8.02 (GraphPad Software, CA, USA).

RESULTS AND DISCUSSIONS

Phytochemical Analysis

Table 1 showed that the crude ethanolic extract and n-hexane, ethyl acetate, and ethanol fraction of *C. odorata* leaves contained alkaloids, flavonoids, terpenoids, phenolic, tannins, steroids, and saponins. Based on Thoden et al., 2007 the alkaloids found in this plant are thought to be 1,2-dehydropyrrolizidine[9]. Alkaloids are secondary metabolites that have the most nitrogen atoms. These compounds are often found in plant and animal tissues. Alkaloids in plants function as metabolites to defend themselves from herbivores and insects[13].

Flavonoids in the leaf extract of *C. odorata* are polar compounds that are easily soluble in polar solvents such as water, methanol, ethanol, butanol. Generally, in plants, flavonoids are bound to sugars, as flavonoid aglycones and glycosides[14]. Several studies state that flavonoid compounds in the ethanolic extract of *C. odorata* leaves function as antioxidants, antimicrobials, anticancer, analgesic, antihepatotoxic, anti-inflammatory, and antihyperglycemic properties[15].

Tannins contain many phenolic hydroxyl groups that enable effective cross-linking with proteins and other molecules such as amino acids, polysaccharides, nucleic acids and fatty acids. Meanwhile, phenol is a compound with analgesic, antimicrobial and anti-inflammatory properties[16].

Table 1. Phytochemical analysis of crude ethanolic extract and fractions of *Chromolaena odorata* Leaves

Phytochemical screening	Fraction			Crude ethanolic extract
	n-Hexane	Ethyl acetate	Ethanol	
Alkaloids				
Mayer's test	-	+	+	+
Dragendroff's test	-	+	+	+
Hager's test	-	+	+	+
Wagner				
Flavonoids				
Bench's Test	+	+	+	+
Shibata's Test	+	+	+	+
Tannins				
Ferric chloride test	-	+	+	+
Lead acetate test	-	+	+	+
Phenolics				
$FeCl_3$	-	+	+	+
Triterpenoids				
Salkowsky test	-	+	+	+
Lieberman-Burchard test	-	+	+	+
Saponins				
Foam test	-	+	+	+
Emulsion test	-	+	+	+
Steroids				
Lieberman-Burchard test	+	+	+	+

LCMS analysis of crude ethanolic extract, n-Hexane fraction, ethyl acetate and ethanol fraction of *Chromolaena odorata* leaves

The results LCMS assay on the crude ethanolic extract of *C. odorata* and its fractions was presented in Table 2. The crude ethanolic extract of *C. odorata* leaves contains the compound 1-Carboethoxy-β-carboline, 3-Methylcanthin- 2, 6- dione, 5,7,8,3′,4′-Pentamethoxyflavonone and Canthin-6-one. In the n-hexane fraction, there are 5,7,8,3′,4′-Pentamethoxyflavonone, Methyl ophiopogonanone A and Pd-C-II compounds.

Meanwhile, in the ethyl acetate fraction, 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one, 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8- methoxy-chroman-4-one, Methyl opiopogonanone A and Sinensetin. The ethanol fraction contained the compound 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one, 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8- methoxy-chroman-4-one and Methyl opiopogonanone A. Neoflavonoids are a group of polyphenolic compounds.

Generally, flavonoids have a 2-phenylchrome-4-one backbone, while neoflavonoids have a 4-phenylchrome backbone without substitution of a hydroxyl group at position 2. The presence of compounds 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one and 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4 -one has proven that this compound belongs to the flavonoid group with a flavonone nuclei structure [17].

Table 2. The results of LCMS analysis of crude ethanolic extract, n-hexane fraction, ethyl acetate and ethanol fraction of *Chromolaena odorata* Leaves

Crude ethanolic extract					
Component name	Observed m/z	Neutral mass (Da)	Observed RT (min)	Adduct	Formula
1-Carboethoxy-β-carboline	241.0969	240.0899	4.31	+H	C14H12N2O2
3-Methylcanthin-2,6-dione	251.0814	250.0742	6.88	+H	C15H10N2O2
5,7,8,3′,4′-Pentamethoxyflavonone	375.1439	374.1366	4.07	+H	C20H22O7
Canthin-6-one	221.0708	220.0637	6.76	+H	C14H8N2O
Candidate Mass C18H16O	271.1079	248.1201	4.7	+Na	C18H16O
n-hexane fraction					
Component name	Observed m/z	Neutral mass (Da)	Observed RT (min)	Adduct	Formula
5,7,8,3′,4′-Pentamethoxyflavonone	375.1437	374.1366	8.04	+H, +Na	C20H22O7
Methyl ophiopogonanone	343.1176	342.1103	8.25	+H	C19H18O6
Pd-C-II	345.1332	344.126	8.74	+H, +Na	C19H20O6
Candidate Mass C18H24O3	289.1798	288.1725	8.34	+H	C18H24O3
Candidate Mass C18H26O3	291.1954	290.1882	9.19	+H	C18H24O4
Ethyl acetate fraction					
Component name	Observed m/z	Neutral mass (Da)	Observed RT (min)	Adduct	Formula
3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one	287.0916	286.0841	8.41	+H	C16H14O5
3-(4’-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4-one	331.1176	330.1103	7.17	+H, +Na	C18H18O6
Methyl ophiopogonanone	343.1175	342.1103	8.26	+H	C19H18O6
Sinensetin	373.1279	372.1209	7.68	+H, +Na	C20H20O7
Candidate Mass C25H46O14	593.2774	570.2888	9.52	+Na	C25H46O14
Ethanol fraction					
Component name	Observed m/z	Neutral mass (Da)	Observed RT (min)	Adduct	Formula
3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one	287.0917	286.0841	8.4	+H	C16H14O5
3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4-one	331.1178	330.1103	7.17	+H, +Na	C18H18O6
Methyl ophiopogonanone	343.1181	342.1103	8.25	+H, +Na	C19H18O6
Candidate Mass C6H11NO3	146.0813	145.0739	1.14	+H	C6H11NO3
Candidate Mass C26H48O14	607.2923	584.3044	9.62	+Na	C26H48O14

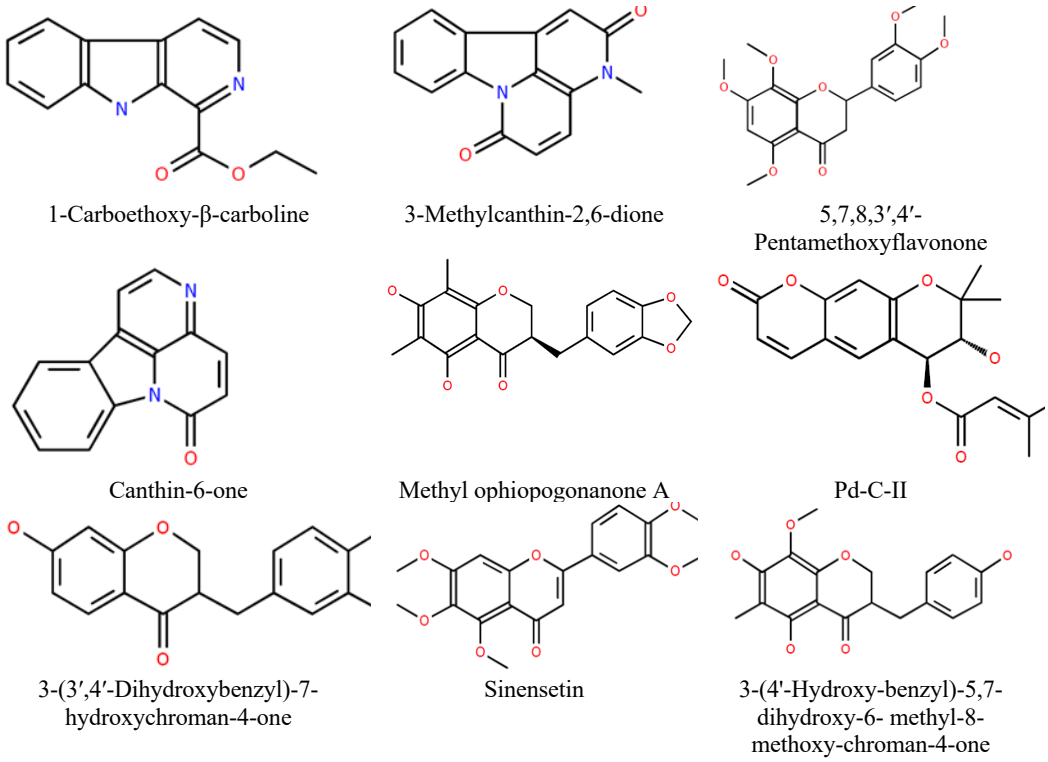

Figure 1. The Compounds in crude ethanolic extract, fraction of n-hexana, ethyl acetate and fraction of ethanol *Chromolaena odorata* Leaves

The anticancer activity of crude ethanolic extract, n-hexane fraction, ethyl acetate and ethanol fraction of *Chromolaena odorata* Leaves on HepG2 cell lines

The IC_{50} values and viability of HepG2 cells are shown in Table 3 and Figure 2. The IC_{50} values of crude ethanol extract and its fractions of *C. odorata* leaves were used as anticancer activity parameters. The IC_{50} value is expressed as the concentration of the extract or fraction that can inhibit the growth of cancer cells by up to 50%[18]. The results showed that the crude ethanolic extract of *C. odorata* leaves is more potent as an anticancer against HepG2 cells with an IC_{50} value of 23.44 g/mL than the n-hexane, ethyl acetate and ethanol fractions (IC_{50}: 84.52µg/mL; 88.51µg/mL; and 167.49 µg/mL, respectively). This may be due to the more complex of components in the crude ethanol extract of *C. odorata* leaves, namely: 1-Carboethoxy-β-carboline, 3-Methylcanthin-2, 6- dion, 5,7,8,3′,4′-Pentamethoxyflavonone and Canthin-6-one. Several researchers stated that synthesized β-carbolin derivatives showed cytotoxic activity with good to moderate potency [19]. A compound of 1-Substituted phenyl-β-carbolin was reported to be a better pharmacophore for cytotoxic activity than the benzo[4.5] canthin-6-one [20]. This is due to having electronically and sterically different nuclei, which may affect various activities and mechanisms of action, mainly related to their lower interactions with DNA, enzymatic systems, or specific receptors for benzo[4,5] canthin-6 -one compared to the 1-substituted phenyl-β-carbolin nuclei [20]. The facts above showed that the diversity of compounds and the potential strength of the tested drug would inhibit the growth of cancer cells with different biochemical reactions.

Table 3. The IC_{50} value of crude ethanolic extract and fractions of *Chromolaena odorata* leaves against HepG2 cancer cell line

Name of tested extract	IC_{50} Value (µg/mL)
Crude ethanolic extract	23.44
N-hexane fraction	84.52
Ethyl acetate fraction	167.49
Ethanol fraction	88.51

In addition, the methoxyflavonones found in the crude ethanolic extract and the n-hexane fraction of the leaves of *C. odorata* (5,7,8,3′,4′ -Pentamethoxyflavonone) are thought to play an essential role in several pharmacological activities, including antiproliferative and apoptosis induction. Other study found that eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone had antioxidant activity, anti-inflammatory, hypoallergenic and

neuroprotective[21-23]. Eupatorin (3′,5-dihydroxy-4′,6,7-trimethoxyflavone) has strong antiproliferative activity against several cancer cells such as prostate cancer [24], cervical (HeLa)[25], human gastric cancer (MK- 1) and human breast cancer cells (MDA-MB-468)[26].

The compounds of 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one and 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4-one in the ethyl acetate fraction and ethanolic fraction have several hydroxyl groups, so it is classified as a flavonoid with a flavonol skeleton[27]. Another study has isolated methylripariochromene-A from the benzene extract of *Eupatorium riparium* [7]. Methylripariochromene-A isolated from the chloroform extract of *E. riparium* had cytotoxic activity against Hela cells with an IC_{50} of 58.32 µg/mL[28]. Other investigators stated that sinensetin exhibits apoptotic effects on hepatocarcinoma cells that is related to p53 expression[29]. In other words, sinensetin induces autophagy in HepG2 cells, p53, including apoptosis in Hep3B cells[29]. Various groups of flavonoids have chemopreventive properties that contribute to the induction of apoptosis by stopping the cell cycle, regulating carcinogen metabolism and oncogenesis expression[30]. The mechanism of action of flavonoids that complement each other in the prevention and therapy of cancer is due to their activity as antioxidants that can bind free radicals, modulate carcinogen metabolism, regulate gene expression on oncogenes and tumor suppressor genes in cell proliferation and differentiation [31]. In addition, flsvonoids can induce apoptosis and cell cycle arrest, modulating enzyme activity in the detoxification process, its anti-inflammatory properties, and its action on other possible targets [31].

Another mechanism of action of flavonoids is influencing the cascade of immunological events associated with cancer development. Understanding the mechanism by which flavonoids can accumulate in organelles and cellular tissues is very important to study because flavonoids can modulate various events in cancer such as apoptosis, vascularization, cell differentiation, and cell proliferation [32]. *In vitro* studies have shown a strong correlation between kinase modulation induced by flavonoids and the incidence of apoptosis, cell proliferation, and invasive behavior of tumor cells. Several flavonoids are also known to have antitumor activity and can suppress the process of angiogenesis by an *in vivo* study [32].

Figure 2 shows that the viability of HepG2 cell decreased in a dose-dependent manner. This indicates that the administration of the test drug in this study could inhibit the cell growth and reduce the viability of HepG2 cells.

The Apoptotic Test on HepG2 Cells by Flowcytometry Method

Cellular apoptosis study using flow cytometry method with Propidium Iodide (PI) and Annexin V has been used to determine whether cells are alive, apoptotic, or necrotic through differences in plasma membrane integrity and permeability.

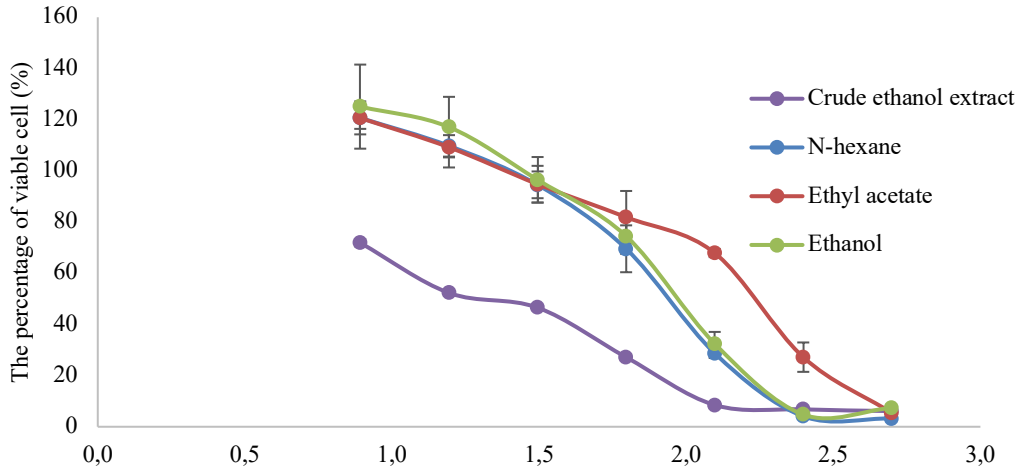

Figure 2. The percentage of viable HepG2 cells after treatment with crude ethanol extract, n-hexane fraction, ethyl acetate, and ethanol fraction of *Chromolaena odorata* leaves with various concentrations for 24 h.

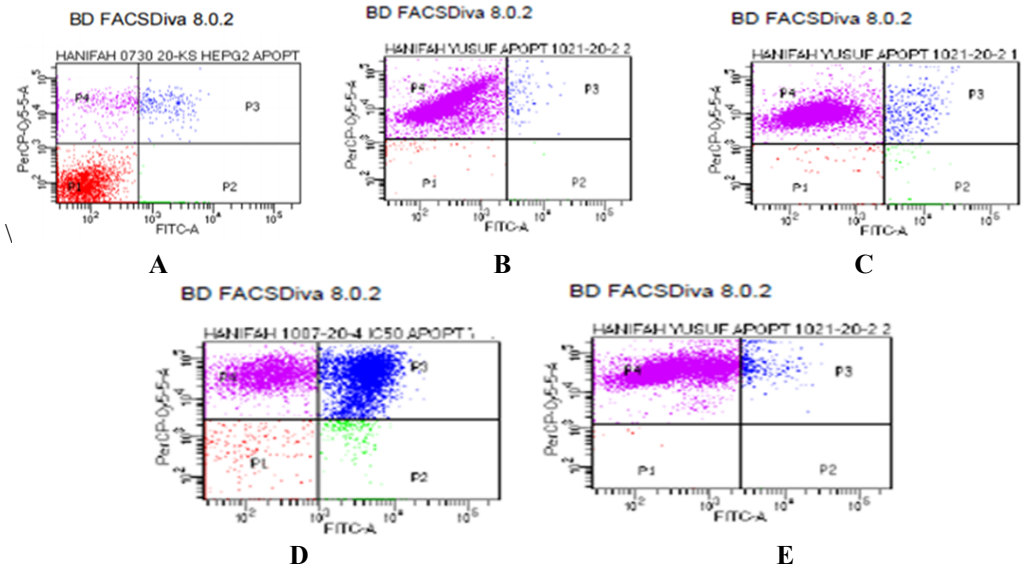

Figure 3. Apoptotic phase of HepG2 cell population after treated with control cells (A = KS, HepG2), the crude ethanol extract (B) and the n-hexane fractions (C), ethyl acetate fraction (D) and ethanol fraction (E) of *Chromolaena odorata* Leaves

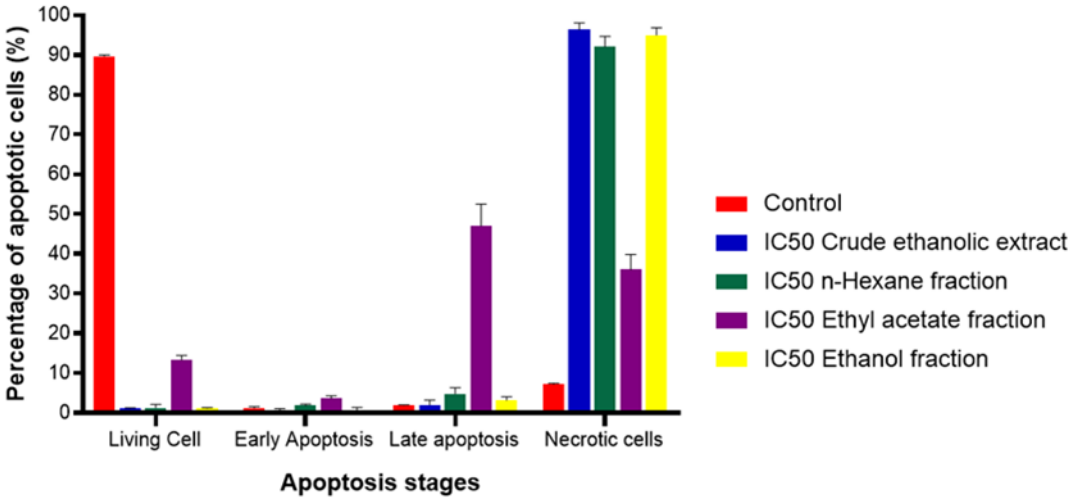

Figure 4. Apoptosis induced by crude ethanolic extract, fraction n-hexane, ethyl acetate and fraction ethanol of *Chromolaena odorata* leaves on HepG2 Cells.

Propidium Iodide does not stain live cells or cells in early apoptotic stages because their plasma membranes are still intact[33]. However, PI will stain apoptotic cells in the late stages and necrotic cells because the integrity of the cell plasma membrane and cell nucleus membrane decreases at this stage. As a result, PI can penetrate the plasma membrane to intercalate into the nucleic acids in the cell nucleus and cause red fluorescence [33].

Figure 3 shows the apoptosis analysis using flowcytometry with HepG2 only as control, and HepG2 treated with IC_{50} of crude ethanolic extract, n-hexane fraction, ethyl acetate fraction, and ethanol fraction of *Chromolaena odorata* Linn as the apoptosis experiments.

Figure 4 shows that the living cells (P1) of control (89.70±0.28%) was significantly higher than all HepG2 cells treated with IC_{50} of all extracts (crude ethanolic extract = 1.15±0.07, fraction n-hexane = 1.3±0.85, ethyl acetate = 13.35±1.06, and ethanol = 1.2±0.14) with p-value < 0.05 in all comparisons. Treatment with IC_{50} of fraction n-hexane has a significantly higher intact cell than IC_{50} of fraction ethyl acetate (p-value<0.05). HepG2 cells undergone early apoptosis after treatment with IC_{50} of ethyl acetate (3.7±0.57%) were significantly higher than HepG2 cells that were

treated with IC_{50} of crude ethanolic extract (0.5±0.57%, p-value<0.001) and fraction ethanol (0.7±0.7%, p-value<0.05). A higher number of HepG2 cells in late apoptosis stage was observed after ethyl acetate treatment (47.0±5.5%) when compared to control and other treatment (p-value<0.05 in all comparisons). Lastly, necrosis cell HepG2 on control (7.2±0.29%) was significantly lower when compared to HepG2 cells treated with crude ethanolic extract (96.3±1.6%), Fraction n-hexane (92.05±2.6%), and ethanol fraction (95.0±1.83%), p-value<0.05 in all comparisons.

The ethyl acetate fraction of *C. odorata* leaves effectively increases the apoptotic process on HepG2 cells. The ability of ethyl acetate fraction to cause apoptosis may be due to the potency of four flavonoids active compounds contained in the ethyl acetate fraction, namely 3-(3′,4′-Dihydroxybenzyl)-7-hydroxychroman-4-one; 3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4-one;Methyl opiopogonanone A and Sinensetin atau 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one[34].

CONCLUSION

Both polar and non-polar compounds in the crude ethanolic and fractions are involved in exerting the anticancer activity of *Chromolaena odorata* Linn, so further analytical studies are needed to identify the responsible compounds and the biochemical pathways for their action. This study also indicated that the chemical composition and pharmacokinetics in the extracts and fractions of *C. odorata* leaves were different.

In this study, the percentage of Hep G2 viable cells decreased in a dose-dependent manner. This indicates that the administration of the tested drugs could inhibit the growth or reduce the viability of Hep G2 cells. Crude ethanol extract of *C. odorata* leaves at low concentration (IC_{50} = 23.44µg/mL) induced cell apoptosis in HepG2 hepatoma cancer cells.

ACKNOWLEDGMENT

We would like to thank the Department of Parasitology, The University of Gadjah Mada for the technical support.

REFERENCES

[1]. Nguyen, K.T. 2011. Targeted nanoparticles for cancer therapy: Promises and challenges. *J. Nanomed. Nanotechnol.* **02**(05).

[2]. American Cancer Society. 2015. Cancer facts & figures 2015. American Cancer Society.

[3]. Carrera, P.M.; Kantarjian, H.M., Blinder, V.S. 2018. The financial burden and distress of patients with cancer: Understanding and stepping-up action on the financial toxicity of cancer treatment. *CA Cancer J. Clin.* **68**(2): 153-165.

[4]. Chui, P.L. 2019. Cancer- and chemotherapy-related symptoms and the use of complementary and alternative medicine. *Asia-Pac. J. Oncol. Nurs.* **6**(1): 4-6.

[5]. Desai, A.G.; Qazi, G.N.; Ganju, R.K.; El-Tamer, M.; Singh, J.; Saxena, A.K.; Bedi, Y.S.; Taneja, S.C.; Bhat, H.K. 2008. Medicinal plants and cancer chemoprevention. *Curr. Drug Metab.* **9**(7): 581-591.

[6]. Muller, P.Y.; Milton, M.N. 2012. The determination and interpretation of the therapeutic index in drug development. *Nat. Rev. Drug Discov.* **11**(10): 751-761.

[7]. Chrystomo, L.Y.; Karim, A.K.; Nugroho, L.H.; Wahyuono, S.; Nohno, T. 2012. Antiproliferative effect of *Eupatorium riparium* reg. Leaves benzene extract against c2c12 and mkn45 cell line in vitro. in International Conference: Research and Application on Traditional Complementary and Alternative Medicine in Health Care (TCAM) Surakarta, Indonesia.

[8]. Antonio, C.N.S.; Selene, M.d.M.; Elnatan, B.d.S.; Raquel, O.d.S.F. 2017. The genus *Eupatorium* l. (asteraceae): A review of their antimicrobial activity. *J. Med. Plants Res.* **11**(3): 43-57.

[9]. Patel, J.; Qureshi, D.; Kumar, S.; Prasad, P.U. 2011. Phytochemical and pharmacological activities of *Eupatorium odoratum* l. *Res. J. Pharm. Technol.* **4**: 184-188.

[10]. Prabhu, V. 2012. Isolation of a novel triterpene from the essential oil of fresh leaves of *Chromolaena odorata* and its in-vitro cytotoxic activity against hepg2 cancer cell line. *J. Appl. Pharm. Sci.* **2**: 132-136.

[11]. Do, Q.D.; Angkawijaya, A.E.; Tran-Nguyen, P.L.; Huynh, L.H.; Soetaredjo, F.E.; Ismadji, S.; Ju, Y.-H. 2014. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *J. Food Drug Anal.* **22**(3): 296-302.

[12]. Iloki-Assanga, S.B.; Lewis-Luján, L.M.; Lara-Espinoza, C.L.; Gil-Salido, A.A.; Fernandez-Angulo, D.; Rubio-Pino, J.L.; Haines, D.D. 2015. Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* l. and *Phoradendron californicum*. *BMC Res Notes*. **8**: 396-396.

[13]. Goyal, S. 2013. Ecological role of alkaloids, in Natural products, K.G. Ramawat and J.-M. Mérillon, Editors., Springer Berlin Heidelberg: Berlin, Heidelberg. p. 149-171.

[14]. Kumar, S.; Pandey, A.K. 2013. Chemistry and biological activities of flavonoids: An overview. *Sci. World J.*: 162750.

[15]. Aziz, N.; Mohamad, M.; Mohsin, H.; Aqmar, N.; Mohamad Nor Hazalin, N.A.; Abdul Hamid, K. 2020. The pharmacological properties and medicinal potential of *Chromolaena odorata*: A review. *Int. J. Pharm. Nutrac. Cosmet. Sci.*

[16]. Huang, Q.; Liu, X.; Zhao, G.; Hu, T.; Wang, Y. 2018. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Anim. Nutr.* **4**(2): 137-150.

[17]. Kaur, H.; Bansal, T. 2020. Role of flavonoids in cancer prevention: Chemistry and mode of action. *Eur. J. Mol. Clin. Med.* **7**(7): 3608-3625.

[18]. Aykul, S.; Martinez-Hackert, E. 2016. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Anal. Biochem.* **508**: 97-103.

[19]. Kontham, V.; Ippakayala, B., and Madhu, D. 2021. Synthesis of β-carboline fatty alcohol hybrid molecules and characterization of their biological and antioxidant activities. *Arab. J. Chem.* **14**(6): 103163.

[20]. Leite Silva, C.M.; Garcia, F.P.; Rodrigues, J.H.; Nakamura, C.V.; Ueda-Nakamura, T.; Meyer, E.; Ruiz, A.L.; Foglio, M.A.; de Carvalho, J.E.; da Costa, W.F.; Sarragiotto, M.H. 2012. Synthesis, antitumor, antitrypanosomal and antileishmanial activities of benzo[4,5]canthin-6-ones bearing the n'-(substituted benzylidene)-carbohydrazide and n-alkylcarboxamide groups at c-2. *Chem. Pharm. Bull.* **60**(11): 1372-1379.

[21]. Lee, M.; Yang, C.; Song, G.; Lim, W. 2021. Eupatilin impacts on the progression of colon cancer by mitochondria dysfunction and oxidative stress. *Antioxidants*. **10**(6): 957.

[22]. Sapkota, A.; Gaire, B.P.; Cho, K.S.; Jeon, S.J.; Kwon, O.W.; Jang, D.S.; Kim, S.Y.; Ryu, J.H.; Choi, J.W. 2017. Eupatilin exerts neuroprotective effects in mice with transient focal cerebral ischemia by reducing microglial activation. *PLoS One*. **12**(2): e0171479.

[23]. Nageen, B.; Sarfraz, I.; Rasul, A.; Hussain, G.; Rukhsar, F.; Irshad, S.; Riaz, A.; Selamoglu, Z.; Ali, M. 2020. Eupatilin: A natural pharmacologically active flavone compound with its wide range applications. *J. Asian Nat. Prod. Res.* **22**(1): 1-16.

[24]. Salmela, A.L.; Pouwels, J.; Kukkonen-Macchi, A.; Waris, S.; Toivonen, P.; Jaakkola, K.; Maki-Jouppila, J.; Kallio, L.; Kallio, M.J. 2012. The flavonoid eupatorin inactivates the mitotic checkpoint leading to polyploidy and apoptosis. *Exp. Cell Res.* **318**(5): 578-592.

[25]. Lee, K.; Hyun Lee, D.; Jung, Y.J.; Shin, S.Y.; Lee, Y.H. 2016. The natural flavone eupatorin induces cell cycle arrest at the g2/m phase and apoptosis in hela cells. *Appl. Biol. Chem.* **59**(2): 193-199.

[26]. Razak, N.A.; Abu, N.; Ho, W.Y.; Zamberi, N.R.; Tan, S.W.; Alitheen, N.B.; Long, K.; Yeap, S.K. 2019. Cytotoxicity of eupatorin in mcf-7 and mda-mb-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis. *Sci. Rep.* **9**(1): 1514.

[27]. Mottaghipisheh, J.; Stuppner, H. 2021. A comprehensive review on chemotaxonomic and phytochemical aspects of homoisoflavonoids, as rare flavonoid derivatives. *Int. J. Mol. Sci.* **22**(5): 2735.

[28]. Fakhruddin, N. 2006. Skrining senyawa sitotoksik dari tumbuhan asal taman nasional gunung merapi yogyakarta:: Kajian terhadap eupatorium riparium reg.(# 04-ku-01-06). Universitas Gadjah Mada.

[29]. Kim, S.M.; Ha, S.E.; Lee, H.J.; Rampogu, S.; Vetrivel, P.; Kim, H.H.; Venkatarame Gowda Saralamma, V.; Lee, K.W.; Kim, G.S. 2020. Sinensetin induces autophagic cell death through p53-related ampk/mtor signaling in hepatocellular carcinoma hepg2 cells. *Nutrients*. **12**(8): 2462.

[30]. Kopustinskiene, D.M.; Jakstas, V.; Savickas, A.; Bernatoniene, J. 2020. Flavonoids as anticancer agents. *Nutrients*. **12**(2): 457.
[31]. Panche, A.N.; Diwan, A.D., and Chandra, S.R. 2016. Flavonoids: An overview. *J. Nutr. Sci.* **5**: e47.
[32]. Batra, P. and Sharma, A.K. 2013. Anti-cancer potential of flavonoids: Recent trends and future perspectives. *3 Biotech*. **3**(6): 439-459.
[33]. Rieger, A.M.; Nelson, K.L.; Konowalchuk, J.D.; Barreda, D.R. 2011. Modified annexin v/propidium iodide apoptosis assay for accurate assessment of cell death. *J. Vis. Exp.*,(50): 2597.
[34]. Yusuf, H.; Fahriani, M.; Murzalina, C. 2022. Anti proliferative and apoptotic effect of soluble ethyl acetate partition from ethanol extract of *Chromolaena odorata* linn leaves against hela cervical cancer cell line. *Asian Pac. J. Cancer Prev.* **23**(1): 183-189.