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Toxicity Analysis of Flavonoid Compounds in Majapahit Leaf Extract (*Crescentia cujete*) Against Various Cancer Cell Lines Using CLC-pred Tools Program

Fatimah[1*], Rahma Diyan Martha[2] and Danar[3]
[1]Department of Medical Laboratory Technology, STIKES Karya Putra Bangsa, 66291, Tulungagung, Indonesia
[2]Department of Pharmacy, STIKES Karya Putra Bangsa, 66291,Tulungagung, Indonesia
[3]Department of Chemistry, Faculty of Mathematics and Science, 65145 Universitas Negeri Malang, Indonesia
*Corresponding author (e-mail: fatimah@stikes-kartrasa.ac.id)

The Majapahit plant (*Crescentia cujete*) is one of the plants that originated from Central America, now widely encountered in Indonesia. Utilization of the Majapahit plant (*Crescentia cujete*) in Indonesia is still very limited, even though in its home country this plant was used as one of the ingredients of traditional medicine. One of the plant organs that is often used is the leaf. Based on this fact, we conducted a study to determine the potential of Majapahit leaves as an anticancer agent. In this study, weanalyzed flavonoid compounds in Majapahit leaves extract (*Crescentia cujete*) using LCMS followed byinsilico analysis of flavonoid compounds with various cancer cell lines using the CLC-pred tools program. Based on compound analysis using LCMS, there are 10 types of compounds in Majapahit leaves extract (*Crescentia cujete*) that are included in the flavonoid group, namely; Quercetin, Acacetin 7- rutinoside, Kaemferol 3-O rhamnoside, Kaemferol 3- [6"-(3- hydroxy- 3- methylglutaryl)glucoside], Kaemferol 3-[6"-(3- hydroxy-3-methylglutaryl)glucoside]-7-glucoside, Didymin, Diosmin, Hesperidin, Narirutin 4'- glucoside, Rutin. The flavonoid compound that had the largest percentage was Kaemferol 3-O rhamnoside at 3.90 percent. The identified flavonoid compounds were analyzedinsilico using the CLC-pred tools program to determine the ability of the compounds to be toxic to cancer cell cultures. Based oninsilico analysis using CLC-pred tools, it is known that each of the identified anticancer potential compounds has different cytotoxic abilities against different types of cell lines.

Keywords: Majapahit leaves; LCMS; anticancer; insilico

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The Majapahit plant (Crescentia cujete L.) is one of the most common plants found in Indonesia, and this plant is native to North America (Burkill, 1985). In Indonesia, the use of this plant is very minimal, and Indonesia people mostly planting this plant as a shade, and some grow wild. Along with the development of science, especially regarding the development of anti-cancer drugs based on natural ingredients, the Majapahit plant began to be investigated further for its potential as one of the natural ingredients that can be developed as an anticancer drug.

Based on (Fatimah et al., 2020) it has been known that the Majapahit plant contains phenolic compounds, flavonoids, alkaloids, and terpenoids. Most of the flavonoid and phenolic compounds are known to have antioxidant activity.Antioxidants are known to protect the body from free radicals is one of the causes of cancer.

In this study, an analysis of the toxicity of flavonoid compounds contained in the leaves of the Majapahit plant against cancer cell lines was carried out insilico using the Way2drung CLC program. So, later, the activity of flavonoid compounds in Majapahit leaves against cancer cells will be known, as well as the potential of Majapahit plant leaves as natural anticancer candidates

EXPERIMENTAL

Materials

This research was carried out using the leaves of the Majapahit plant taken from the yard of STIKes Karya Putra Bangsa, Tulungagung, and 96% ethanol as a solvent purchased from Merck.

Making the Ethanol Extract of the Majapahit (*Crescentia cujete*) Plant

The leaves of the Majapahit plant were extracted using the maceration method with 96% ethanol solvent. The maceration process were begun by taking the leaves of the Majapahit plant. The leaves of the plants that have been taken are then washed and dried.

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Then the dry leaves are crushed using a blender. The crushed leaves were weighed as much as 100 grams and macerated using 96% ethanol solvent. The maceration results are then evaporated using a rotary evaporator, so that a thick extract is obtained. The extract was then analyzed for its compound content using the LCMS device.

Compound Analysis of the Ethanol Extract of the Majapahit (*Crescentia cujete*) Plant using LCMS

The Majapahit plant leaves ethanol extract compounds were identified by using LCMS (Liquid Chromatography Mass Spectrometry). The LCMS process was carried out by injecting a sample of Majapahit leaf extract that had been prepared into the LCMS-8040 instrument, Shimadzu brand. LCMS (Liquid Chromatography Mass Spectrometry) analysis was performed with UPLC-MS (Ultra Performance Liquid Chromatography-Mass Spectrometry) equipped with a binary pump. LC (Liquid Chromatography) connected Quadrupole Time-of-Flight (QTOF) mass spectrometer equipped with an Electrospray Ionization (ESI) ionization source. Mass spectrometry (MS) that was used is the QTOF system with positive ionization mode. The ESI (Electrospray Ionization) parameters that was used include a capillary temperature of 350 C and an atomizer gas of 60ML/ HR, with a voltage source of 5.0V. A full scan mode from m/z 100-5000 was performed with a source temperature of 100 C. UPLC column used Shimadzu Shim Pack FC-ODS (2mm x 150mm, 3µm). The eluent used was 90% methanol and water. The eluent adjusted at a total flow rate of 0.5 ml/min (Fatimah et al., 2020). The results of all identified compounds were then classified by referring to the PubChem database.

Toxicity Analysis of Detected Flavonoid Compounds Towards Cancer Cell Line using Insilico Methods

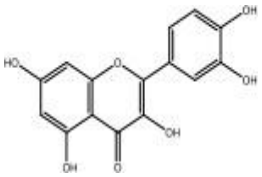
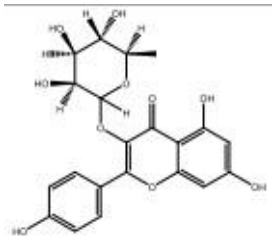
The flavonoid compounds identified by LCMS were then analyzed for their toxicity to cancer cell line insilico using the Ways2drugsCLC program. Toxicity analysis of the compounds was carried out by entering the structure of the flavonoid compounds found in the extract and performing a cytotoxic analysis of cancer cell lines with a pa>pi value with a threshold of pa>0.5 (Lagunin et al., 2018).

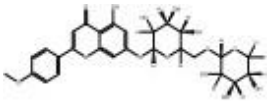
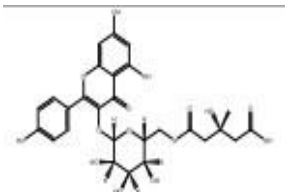
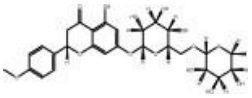
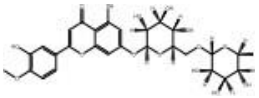
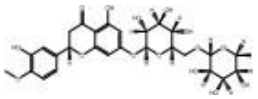
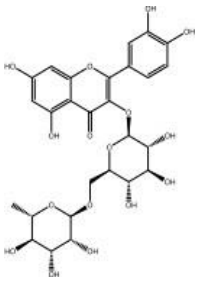
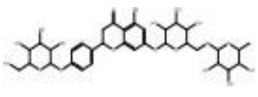
RESULTS AND DISCUSSION

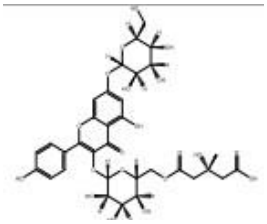
Identification of Potential Anticancer Flavonoid Compounds in Majapahit Plant Leaf Extract

Based on the result of LCMS detection, there were approximately 97 compounds detected. The compounds were distributed into the major group, such as; terpenoid, alkaloid, coumarin, and flavonoid. In this research, we did a thorough analysis of the compounds included in flavonoid groups. We mainly focus on flavonoid because the compounds that are included in the flavonoids group mostly has anticancer activity, so they can be developed as a source of anticancer. Here is the activity of flavonoids as an anti-cancer, such as; inducing apoptosis (Kajimoto et al., 2002), elevating the expression of p53 that acted as a tumor suppressor gene (Gu et al., 2022), modulating cellular ROS (Slika et al., 2022), inhibit cancer cell proliferation (Park et al., 2022), and modulating transduction signal to inhibit angiogenesis (Al Jumaili and Al Hdeethi., 2021).The list of secondary metabolites of the flavonoid group found in the leaf extract of the Majapahit plant (Crescentia cujete L.) using LCMS can be saw in (table 1.).

Table 1. Flavonoid compound detected in Majapahit leaves extract.

No	Retention Time (min)	Peak Number	Compound	Composition (%)	Structure
1.	11,427	67	Quercetin Chemical Formula: $C_{15}H_{10}O_7$ Exact Mass: 302,0427 Molecular Weight: 302,2380 m/z: 302.0427 (100.0%), 303.0460 (16.2%), 304.0469 (1.4%), 304.0494 (1.2%)	1,97	
2.	21,429	84	Kaempferol-3-O-rhamnoside Chemical Formula: $C_{21}H_{20}O_{10}$ Exact Mass: 432,1056 Molecular Weight: 432,3810 m/z: 432.1056 (100.0%), 433.1090 (22.7%), 434.1124 (2.5%), 434.1099 (2.1%)	3,90	

3.	33,072	86	Acacetin 7-rutinoside Chemical Formula: $C_{28}H_{32}O_{14}$ Exact Mass: 592,1792 Molecular Weight: 592,5500 m/z: 592.1792 (100.0%), 593.1826 (30.3%), 594.1859 (4.4%), 594.1835 (2.9%)	2,70	
4.	33,729	88	Kaempferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside] Chemical Formula: $C_{27}H_{28}O_{15}$ Exact Mass: 592,1428 Molecular Weight: 592,5060 m/z: 592.1428 (100.0%), 593.1462 (29.2%), 594.1471 (3.1%), 594.1495 (2.7%), 594.1495 (1.4%)	3,19	
5.	34,004	89	Didymin Chemical Formula: $C_{28}H_{34}O_{14}$ Exact Mass: 594,1949 Molecular Weight: 594,5660 m/z: 594.1949 (100.0%), 595.1982 (30.3%), 596.2016 (4.4%), 596.1991 (2.9%)	1,70	
6.	35,504	90	Diosmin Chemical Formula: $C_{28}H_{32}O_{15}$ Exact Mass: 608,1741 Molecular Weight: 608,5490 m/z: 608.1741 (100.0%), 609.1775 (30.3%), 610.1808 (4.4%), 610.1784 (3.1%)	1,84	
7.	35,507	91	Hesperidin Chemical Formula: $C_{28}H_{34}O_{15}$ Exact Mass: 610,1898 Molecular Weight: 610,5650 m/z: 610.1898 (100.0%), 611.1931 (30.3%), 612.1965 (4.4%), 612.1940 (3.1%)	3,77	
8.	35,517	92	Rutin Chemical Formula: $C_{27}H_{30}O_{16}$ Exact Mass: 610,1534 Molecular Weight: 610,5210 m/z: 610.1534 (100.0%), 611.1567 (29.2%), 612.1576 (3.3%), 612.1601 (2.7%), 612.1601 (1.4%)	3,43	
9.	46,301	96	Narirutin 4’-glucoside Chemical Formula: $C_{33}H_{42}O_{19}$ Exact Mass: 742,2320 Molecular Weight: 742,6800 m/z: 742.2320 (100.0%), 743.2354 (35.7%), 744.2387 (6.2%), 744.2363 (3.9%), 745.2396 (1.4%)	2,21	

10.	46,564	97	Kaemferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside]-7-glucoside Chemical Formula: $C_{33}H_{38}O_{20}$ Exact Mass: 754,1956 Molecular Weight: 754,6470 m/z: 754.1956 (100.0%), 755.1990 (35.7%), 756.2024 (6.2%), 756.1999 (4.1%), 757.2032 (1.5%)	2,60	

Based on (Table 1), it can be saw that there are about ten compounds belonging to the flavonoid group in the leaf extract of the Majapahit plant. The flavonoid compound with the largest percentage was kaempferol 3-O rhamnoside at 3.90%. As for knowing the anticancer potential of the flavonoid compounds that have been identified, the comparisons were made from the Pub-Chem database and literature studies from several journals. Based on data analysis and literature study, it has been known that all of the flavonoid compounds contained in the leaf extract of the Majapahit plant have the potential as an anticancer.

Quercetin was known to induce apoptosis in thyroid cancer by inducing the expression of the pro-NAG-1 protein, which has an antitumor role (Hong et al., 2021). In addition to thyroid cancer, quercetin loaded with PLGA nanoparticles (PLGA- QNPs) is known to decrease the viability of human cervical cancer cells and breast cancer cells (Yadav et al., 2022). Kaemferol 3-O rhamnoside derived from leaf extract of Schima wallichii Korth can inhibit the growth of MCF-7 breast cancer cells (Diantini et al., 2012). Kaemferol 3-O rhamnoside found in the leaf extract of the Etligera elaitor plant is known to inhibit HeLa cell proliferation (Herni et al., 2021). Glycosylated kaempferol, such as; Kaempferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside] and Kaempferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside]-7-glucoside are known to acts as an antiproliferative agent in hepatocarcinoma cells (Imran et al., 2019). Acacetin 7-rutinoside (linarin) can increase antiproliferative activity in human non-small cell lung cancer (Thaipong et al., 2006; Mottaghipisheh et al., 2021). This anti-proliferative activity occurs through suppression of Akt activation and induction of the cyclin-dependent kinase inhibitor p27Kipl (Thaipong et al., 2006). Didymin is known to have the ability as an anti-angiogenic agent by prevent the VEGF from inducing cell proliferation (Shukla et al., 2019). Angiogenesisis one of the hallmarks of cancer. Thus, if angiogenesis can be prevented, it is hoped that the development of cancer cells can be inhibit. Diosmin is known to reduce the growth of HepG2 liver cancer cells (Cheng et al., 2021). In addition, diosmin is also known to induce apoptosis in human glioblastoma cells (Soares et al., 2019). Hesperidin is known to suppress cell replication in the HEp-2 laryngeal carcinoma cell lines, by triggering apoptosis (El Wahed et al., 2022). Hesperidin is also known todecrease cell proliferation and migration, in two types of oral cancer cell lines (HN6 and HN15) (Wudtiwai et al., 2021). Rutin is known to affect signaling pathways in apoptosis (Satari et al., 2021). Narirutin 4'-glucoside and several types of flavonoid compounds that contained in citrus fruit peels can inhibit the growth of HL-60 cell cultures (Diab et al., 2015).

Cytotoxicity Analysis of Flavonoid Compounds Against Cancer Cell Lines using the Way2-drugs CLC Program

In silico analysis of the toxicity of compounds can be determined using several applications, one of which is Way2drugsCLC. This program can be used to predict the toxicity of compounds to both cancerous and normal cell line cultures. However, this study focused on understanding the effect of toxicity on cancer cells. In (Table 2), the reaction of flavonoid compounds detected in the extract of the leaves of the Majapahit plant with cancer cell lines can be seen.

Based on (Table 2), it can be seen from a total of 10 flavonoid compounds detected using LCMS, only eight compounds are known to have cytotoxic activity on cancer cells. Even so, it does not mean that the other two compounds do not have anticancer activity. Both compounds still have anticancer activity even though the toxicity value is low. That is because the toxicity analysis was carried out with a threshold of pa>0,5 if it was carried out with a threshold of pa<0.5, all compounds known to have cytotoxic activity against cancer cell lines. In this study, to minimize the occurrence of false positives, the cytotoxicity analysis was carried out using a threshold pa>0.5. The greater the threshold pa, the smaller the false positive (Lagunin et al., 2018). In (Table 2), it can be seen that several compounds appear to have cytotoxic activity against the same types of cells, including HL-60, SK-MEL-1, and NCI-H838. There are three compounds that have cytotoxic activity against HL-60, namely; Acacetin 7-rutinoside, Diosmin, and Narirutin 4'-glucoside. The compound that has the highest cytotoxic activity against HL-60 was Narirutin 4'-glucoside with a pa value> 0.559. There are four compounds that have cytotoxic activity against SK-MEL-1, namely; Kaemferol-3-O-rhamnoside,Kaemferol3-[6”-(3-hydroxy-3-methylglutaryl) glucoside], Rutin, and Kaemferol 3-[6”-(3-hydroxy-3-methylglutaryl) gluco-side]-7-glucoside. The compound that has the highest

Table 2. Cytotoxicity analysis of anticancer potential compounds towards cancer cellline.

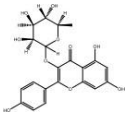
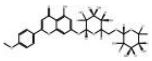
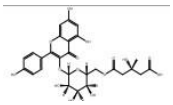
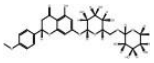
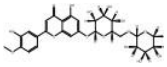
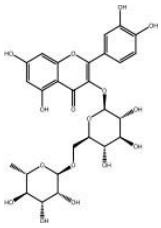
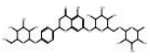
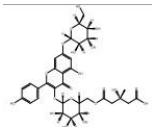
Compounds	Structure	Cell line	Cell line model type	Affecting parts	Tumor type	Pa	Pi
Kaemferol-3-O-rhamnoside		SK-MEL-1	Metastatic melanoma	Skin	Melanoma	0,536	0,020
Acacetin 7-rutinoside		NCI-H838	Non-small cell lung cancer.3 stage	Lung	Carcinoma	0,553	0,028
		HL-60	Promyelo blast leukimia	Haematopoietic and lymphoid tissue	Leukemia	0,535	0,023
Kaemferol 3-[6”-(3-hydroxy-3-methylglutaryl)glucoside]		SK-MEL-1	Metastatic melanoma	Skin	Melanoma	0,523	0,024
Didymin		NCI-H838	Non-small cell lung cancer.3 stage	Lung	Carcinoma	0,534	0,034
Diosmin		Caco-2	Colon adenocarcinoma	Colon	Adeno carcinoma	0,651	0,003
		HL-60	Promyeloblast leukimia	Haematopoietic and lymphoid tissue	Leukemia	0,526	0,024
		NCI-H838	Non-small cell lung cancer.3 stage	Lung	Carcinoma	0,528	0,036
Rutin		SK-MEL-1	Metastatic melanoma	Skin	Melanoma	0,558	0,013
Narirutin 4’-glucoside		NCI-H838	Non-small cell lung cancer.3 stage	Lung	Carcinoma	0,559	0,026
		HL-60	Promyelo blast leukimia	Haematopoietic and lymphoid tissue	Leukemia	0,553	0,021
Kaemferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside]-7-glucoside		SK-MEL-1	Metastatic melanoma	Skin	Melanoma	0,523	0,024

Table 2. detailed note; Pa: Probable activity, Pi: Probable inactivity

cytotoxic activity against SK-MEL-1 was rutin with a value of pa>0.558. There are four compounds that have cytotoxic activity against NCI-H838, namely; Acacetin 7-rutinoside, Didymin, Diosmin, and Narirutin 4'-glucoside. The compound that had the highest cyto-toxic activity against NCI-H838 was Narirutin 4'-glucoside with a pa value > 0.559. The validity of the results of the analysis using Way2drugsCLC can be said to be very high (Lagunin et al., 2018). However, it is necessary to conduct further studies on the compound toxicity to cancer cells in-vitro, because there are many factors that can affect the growth of cancer cell lines in the laboratory.

CONCLUSION

Based on the research that has been done, it can be seen that in the extract of the leaves of the Majapahit plant (Crescentia cujete) there are about ten types of flavonoid compounds. Based on the analysis of the toxicity of compounds using the Way2drugsCLC program with a threshold of pa>0.5, it is known that there are about eight compounds out of a total of 10 compounds that are toxic to several types of cancer cells.

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