MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2025.20.1.13579

Kojic Acid from the Ethyl Acetate Fraction of *Terminalia catappa* Linn Fruit Flesh and its anti-cancer activity against HeLa cells

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Received November 11, 2024; **Accepted** January 31, 2025; **Available online** March 20, 2025

ABSTRACT. The ethyl acetate fraction of *Terminalia catappa* Linn. fruit flesh has anti-cancer activity against HeLa cells, with IC_{50} value is =165.37 ppm. The aim of this research is to isolate active anticancer compound from the ethyl acetate fraction of *T. catappa* fruit flesh. Anti-cancer active compounds from the ethyl acetate fraction of *T. catappa* fruit flesh were purified using column chromatography and continued with recrystallization. The compound isolate from the ethyl acetate fraction is clear yellowish crystals and has a melting point 150 ± 2 °C, has anti-cancer activity against HeLa cells of IC_{50} =413.695 ppm. Results of molecular structure identification using UV spectroscopy; FTIR; ^{1}HNMR, ^{13}CNMR and LC-MS are thought to be the compound 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4- one (Kojic acid) with a molecular weight of 142.36 gram/mol. The Kojic acid content in *T. catappa* Linn fruit flesh was found to provide information on the potential use of *T. catappa* fruit flesh extract in the pharmaceutical and cosmetics industries.

Keywords: Anticancer activity, HeLa cells, Kojic acid, *T. catappa* Linn,

INTRODUCTION

Cancer treatments such as chemotherapy, radiotherapy and irradiation therapy have side effects that can reduce quality of life and lead to complications. An important step for cancer treatment is chemotherapy and radiotherapy. However, the effect of resistance to radiotherapy and chemotherapy will actually result in cancer recurrence. Radioresistance in cancer is often caused by a repair response radiation-induced DNA damage, cell cycle dysregulation, cancer stem cells (CSC) resistance, and epithelial-mesenchymal transition (Liu et al., 2021).

Continuous research to find anticancer drugs includes looking for compounds from natural resources. Exploring the potential of natural resources containing anticancer bioactive compounds by screening and isolating bioactive compounds (Hosseini & Ghorbani, 2015). The discovery of anticancer drugs from natural ingredients is increasingly necessary. The reasons include the large diversity of molecules and their potential efficacy and safety as medicinal ingredients due to the uniqueness of the molecules (Naeem et al., 2022). Besides that, medicines made from natural ingredients are safer and have minimal side effects (Purwaningsih et al., 2020). One of the compounds derived from natural materials is kojic acid. Kojic acid (5- hydroxy- 2- hydroxymethyl- 4H- pyran- 4- one) is an organic acid derived from the fermentation of fungi of diverse genus such as Aspergillus and Penicillium (Zilles et al., 2022). The major applications of kojic acid and its derivatives in medicine are based on their biocompatibility, antimicrobial and antiviral, antitumor, antidiabetic, anticancer, anti-speck, anti-parasitic, and pesticidal and insecticidal properties (Saeedi et al., 2019).

Testing the anti-cancer effect of of natural sources can be done through a cytotoxic test using a cancer cell model. There are many cell models that can be used such as HeLa, RAJI, K562, NB4, MCF-7, Vero and so on (Lukman et al., 2021; Mirabelli et al., 2019). The use of HeLa cells has been widely used in biomedical research and medicine. HeLa cells are HPV-18 type cervical cancer cells. For the purposes of testing the anticancer activity of a material, the MTT method is used. The MTT method is a method carried out using a colorimetric test which is based on changing tetrazolium salt to formazan in active mitochondria in living cells (Rai et al., 2018).

Previous research has tested several plants which have been empirically declared to have anticancer properties. One of them is *Terminalia catappa* Linn (Katili et al., 2015). The extract of *T. catappa* leaves and fruits have anticancer, antioxidant, anti-HIV

reverse transcriptase, anti-inflammatory, antidiabetic effects and hepatoprotective activities (Mohale et al., 2009). Research conducted by (Tampemawa et al., 2016) on *T.catappa* leaves as an antibacterial showed that they had an inhibitory effect on the bacteria *B.amyloliquefaciens*. The results of research by Widyaningsih, et al obtained information that *T.catappa* fruit flesh extract was toxic to Artemia salina Leach shrimp larvae with an LC_{50} value were 17.171 ppm (Widyaningsih et al., 2022). The phytochemicals screening showed that ethyl acetate extract of the *T.catappa* fruit flesh contained flavonoid, alkaloid, phenolic, terpenoid, and saponin compounds (Widyaningsih et al., 2022). The results of other studies indicate that *T. catappa* is thought to contain potential antitumor compounds and could be a candidate for further research (Zarredar et al., 2021). The content of flavonoid compounds in *T.catappa* fruit is mucic acid (l), quercetin 7-O-rhamnoside, limocitrin, rottlerin and several other flavonoid compounds (Venkatalakshmi et al., 2016). Flavonoid group compounds found in methanol extract of *T.catappa* fruit are apigenin and quercetin groups (Yakubu et al., 2021). Alkaloid group compounds identified in acetone extract of *T.catappa* leaves, one of which is anhalonidin ($C_{12}H_{17}NO_3$) (Sowmya & Raveesha, 2021). Phenolic acid group compounds identified in *T. catappa* leaves are gallic acid ($C_7H_6O_5$) (Yakubu et al., 2021). One of the terpenoid compound derivatives identified in *T. catappa* leaves is 3-Oxo-12,18-ursadien-28- oic acid ($C_{30}H_{44}O_3$) (Sowmya & Raveesha, 2021). Steroid class compounds identified in *T. catappa* fruit seed oil are cholesterol ($C_{27}H_{46}O$) (Ladele et al., 2016). The main compound of the tannin group in *T. catappa* leaf extract is punicalagin (Mininel et al., 2014).

Chasani, et al, 2022 obtained the active anticancer fraction from the results of column chromatography of the ethyl acetate fraction of *T. catappa* fruit flesh. The ethyl acetate fraction was obtained from the fractionation of the methanol extract of *T.catappa* fruit flesh. The first fractionation used *n*-hexane and continues with ethyl acetate. Results of column chromatography of the ethyl acetate fraction of *T. catappa* fruit flesh obtained 7 groups of fractions (Chasani et al., 2022). Based on the results of the toxicity test of the seven fractions, data was obtained that there was one fraction that had the highest toxicity to shrimp larvae *A. Salina* Leach, namely 87.91 ppm. The results of testing on HeLa cells showed an IC_{50} activity value of 165.37 ppm (Chasani et al., 2022). To obtain active anticancer compounds from the ethyl acetate fraction of *T. catappa* fruit flesh, a compound purification step is required. One technique for purifying compounds is the gravity column chromatography method. Gravity column chromatography is a versatile separation technique and is widely used in the field of chemistry, especially the separation of different compounds based on their affinity for the stationary phase and their interaction with the mobile phase (Battistini, 2023). Therefore, to obtain anticancer active compounds from the ethyl acetate fraction of *T. catappa* fruit flesh, a purification process was carried out from the fraction of column chromatography results that had the highest anticancer activity. Purification was carried out by column chromatography and recrystallization techniques. The purified isolate compound was characterized and its molecular structure was identified.

This article discusses the results of purifying active anticancer compounds from the ethyl acetate fraction of *T. catappa* fruit flesh. The gravitational column chromatography and recrystallization method was used to obtain pure compounds. The isolated compound obtained was tested for its activity against HeLa cells and its molecular structure was determined using UV, FTIR, LCMS, ^{1}HNMR and ^{13}CNMR spectroscopy. To streng then the alleged molecular structure, the spectrum data obtained was compared with the spectrum data of previously discovered compounds. It is hoped that the results of this research will provide additional information regarding the potential of *T. catappa* fruit flesh as an anti-cancer agent and the molecular structure of the active anti-cancer compounds obtained.

EXPERIMENTAL SECTION

Material

The materials used were the ethyl acetate extract of *T. catappa* fruit flesh obtained from Purwokerto, Banyumas Regency, Central Java, Indonesia, methanol, n-hexane, ethyl acetate, chloroform, acetic acid, G_{60} silica gel, G_{254} TLC plate. All materials are from E. Merck.

Purification of Ethyl Acetate Fraction

The ethyl acetate fraction of *T.catappa* fruit pulp was subjected to column chromatography using the mobile phase methanol: ethyl acetate: chloroform: glacial acetic acid (0.5:3:8:0.1). The purity test of the fraction resulting from column chromatography was carried out using thin layer chromatography (TLC) on a similar eluent. Fractions that have one spot are combined and dried then tested for purity using TLC. The compound purification process is carried out using the recrystallization technique: The sample was dissolved in a mixture of ethyl acetate: methanol (1:4) and heated slowly while stirring until all components of the compound were completely dissolved. This solution was left at room temperature until crystals formed. Crystals are separated by filtration. The resulting crystals were dissolved in methanol: ethyl acetate (1:4) and eluted using TLC with the eluent methanol: ethyl acetate: chloroform: glacial acetic acid (0.5:3:8:0.1). The TLC spots obtained were observed using a 254 nm and 366 nm UV lamp. If there are still impurity spots, re-purification is carried out continuously until one pure compound.

The pure compounds obtained were tested for melting point, solubility and anticancer activity against HeLa cells using MTT method. Identify the molecular structure using UV, FTIR, 1HNMR, 13CNMR and LCMS.

Anticancer Activity Test Against HeLa Cells Using MTT Method

Prepare an MTT solution by dissolving 5 mg of MTT powder in 1 mL of PBS solution. Added to the MTT solution were 10 exp 4 cells and 100 µL of culture medium into each 96 well plate. Three replicates were used for each concentration. Then the cells were incubated in 5% CO_2 at 37 degrees Celsius for 12 hours with the aim of allowing the cells to stick to the bottom of the dish. After the cells attach, the cells are treated with various concentrations of the compounds tested and then incubated for 24 hours. After the treatment period was complete, the media was discarded, then 100 μL of culture media was poured into each well and continued with the addition of 10 μL of MTT solution to each well. The well plates were kept for incubation in 5% CO_2 and 37 °C for 3-4 hours. Next, the supernatant was discarded and continued with the addition of 100 μL of isopropanol or DMSO detergent to dissolve the formazan crystals resulting from MTT. Homogenization is carried out by shaking the well-plate slowly for 10-15 minutes until an evenly dissolved solution is obtained. Next, the well-plate was read at a wavelength of 570 nm and the percentage of live cells was calculated. Anticancer activity is determined by the IC_{50} value obtained from x. value by substituting y = 50. This value shows a 50% reduction in absorption in the line equation y = mx+b. The equation of the line obtained will create a graph of the relationship between % inhibition and sample concentration.

RESULTS AND DISCUSSIONS

Purification of active anticancer compound from the ethyl acetate fraction of *T. catappa* fruit flesh using gravity column chromatography with a silica gel 60 GF254 stationary phase.The mobile phase used was methanol: ethyl acetate: chloroform: glacial acetic acid (0.5:3:8:0.1). The chromatogram of the ethyl acetate fraction of *T. catappa* fruit flesh, the fraction resulting from column chromatography and the pure compound resulting from recrystallization obtained is shown in **Figure 1**.

The results of the purification obtained one compound in the form of clear yellowish crystals (**Figure 2**) with a yield of 6.30% (w/w) of the weight of the ethyl acetate fraction before purification and having a melting point of 150 ± 2 °C. Based on solubility test results showed that its well soluble in methanol and acetone but less soluble in ethyl acetate and not soluble in *n*-hexane and chloroform.

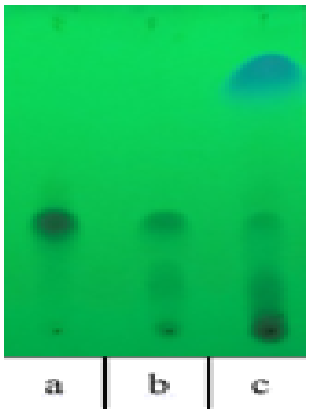

Figure 1. TLC profile of the isolated pure compound (a), Fraction from column chromatography before recrystallization (b), and ethyl acetate fraction of *T. catappa* fruit flesh (c)

Figure 2. Crystal profile of the isolated compound

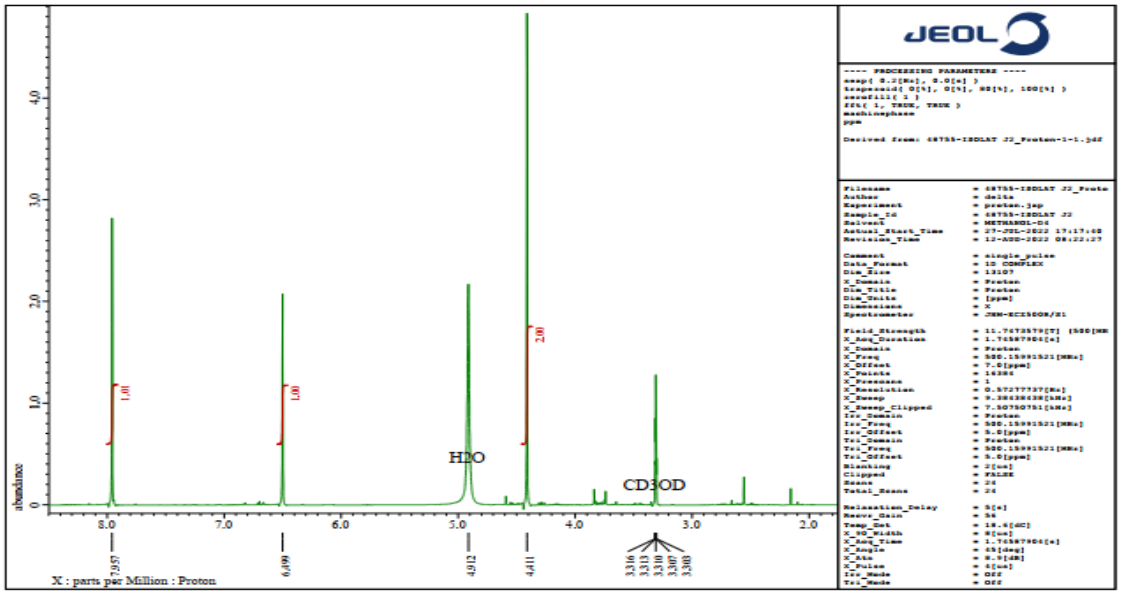

Figure 3. [1]HNMR spectrum of isolated compound

Identification of the molecular structure of the isolated compound using [1]HNMR and [13]CNMR obtained a spectrum as shown in **Figures 3** and **Figure 4.** [1]HNMR spectrum data shows the presence of 2 H signals originating from one singlet methylene signal (δ_H 4.41 ppm,), one proton signal from isolated methine in the pyran system at δ_H 6.50 ppm as well one proton signal from isolated cyclic methine bound to an electron-withdrawing group at δ_H 7.96 ppm. [13]CNMR spectrum shows 6 carbon signals originating from one sp^3 methylene carbon (δC 61.26 ppm), two methine sp^2 carbons in the aromatic system at δ_C 110.82 and 141.11 ppm, two sp^2 quaternary carbons in the aromatic system δ_C 147.50 and 170.56 ppm), and one carbonyl carbon at δ_C 176.08 ppm. Based on chemical shifts of The [1]HNMR and [13]CNMR data of isolated compound was elucidated as kojic acid and supported by the reference data of kojic acid (DellaGreca et al., 2019) presented in **Table 1**.

The results of identifying the isolate compound using LCMS are shown in **Figure 5**. The fragment at m/z 143,36 $[M+H]^+$ is the molecular ion weight of the compound. Other fragments appear at m/z 83 and m/z 60.28. The results of analysis using LCMS indicate the presence of a compound with a molecular weight of = is 142.36 grams/mol and the molecular formula $C_6H_6O_4$. This LCMS spectrum data supports the assumption that the isolated compound is kojic acid. Kojic acid has a molecular weight of 142.11 with the molecular formula $C_6H_6O_4$.

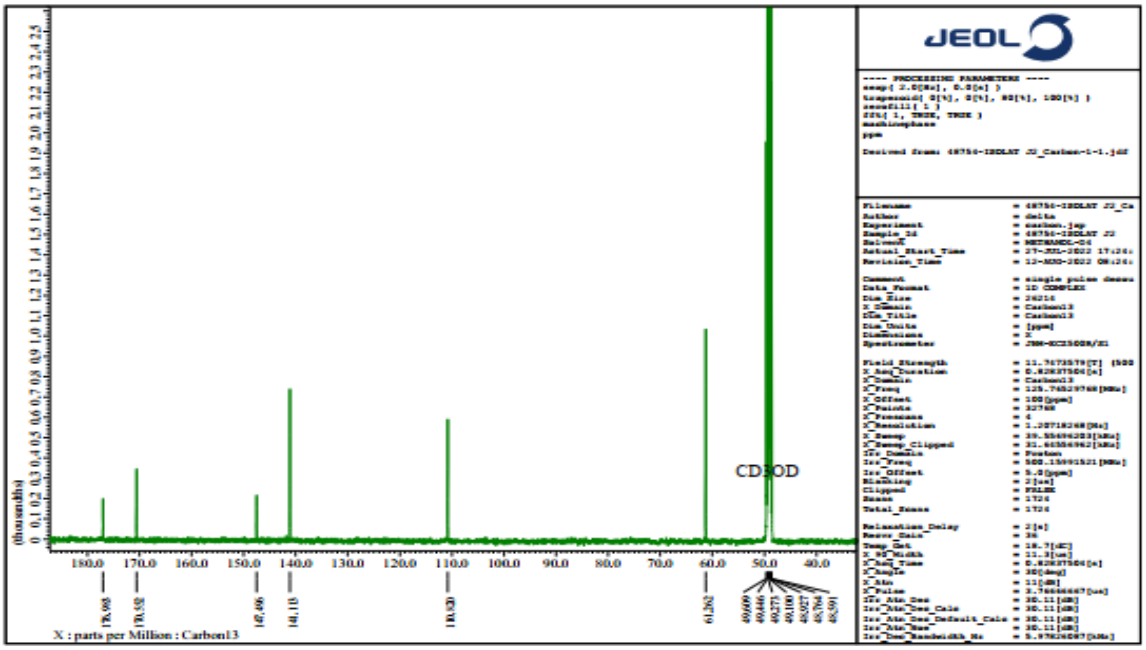

Figure 4. [13]CNMR spectrum of the isolated compound

Table 1. ^{1}HNMR and ^{13}CNMR spectrum data for isolate compounds and spectra reference 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-0ne (kojic acid)

Position of the C atom	δH (mult. J Hz) ppm		δC ppm	
	Isolate compound	kojic acid*	Isolate compound	kojic acid*
1	-	-	170.56	169.3
2	6.50 (1H, s)	6.52	110.82	109.3
3	-	-	176.06	175.5
4	-	-	147.50	146.0
5	7.96 (1D, s)	7.97	141.11	139.6
6	4.41 (2H, s)	4.49	61.26	59.8

* (DellaGreca et al., 2019)

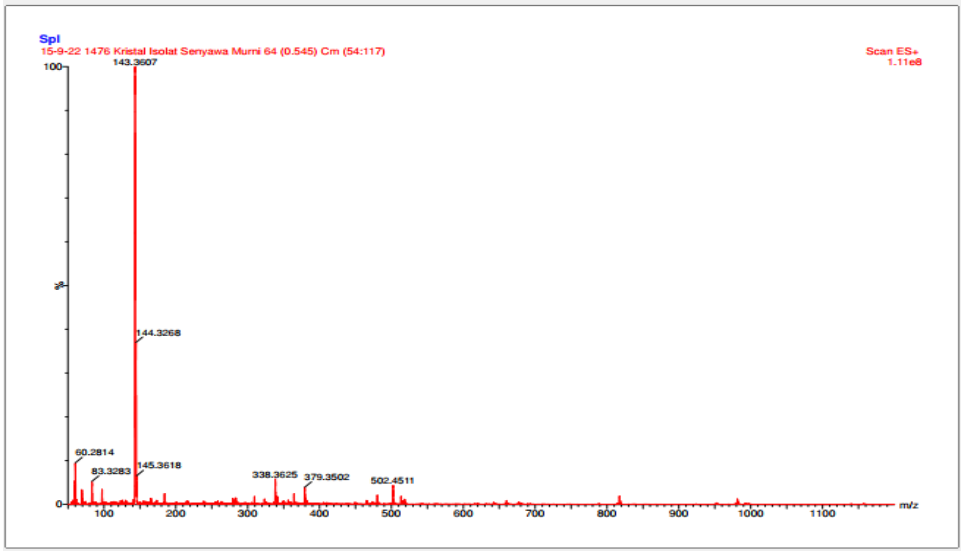

Figure 5. LCMS spectrum of isolated compounds

Identification results using an FTIR spectrophotometer are presented in **Figure 6**. FTIR spectrum data shows that there is an absorption indicating the presence of an ether group, namely at 1234.44 cm-1. The absorption at 3271.27 cm-1 is quite wide, indicating the presence of an alcohol group. The existence of the C=C double bond is also seen in the absorption of 987.55 cm^{-1} which is a substituted alkene. The presence of the C-sp^3 group can be seen at the absorption of 2924.09 cm^{-1}.

Identification results using a UV spectrophotometer showed that the pure isolate contained a chromophore carbonyl group (C=O) at wavelengths of 249.0 nm and 269.50 which was caused by a shift in electrons from the π to π^* orbitals. The spectrum obtained also explains the presence of heteroatoms and conjugation systems in the pure isolate.

Based on ^{1}HNMR, ^{13}CNMR, LCMS, FTIR and Ultra violet spectrum data, it is suspected that the isolate compound is 5-Hydroxymethyl-3-furan-carboxylic acid. This suspicion is strengthened by data on the molecular weight of the isolate compound which is the same as the molecular weight of 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-0ne (kojic acid), namely 142.11 gram/mol. Based on data, the melting point of the isolate compound is 150 ± 2 °C, which is close to the melting point of the 5-Hydroxymethyl-3-furan-carboxylic acid compound, namely 151-154 °C. The compound structure of 5-Hydroxymethyl-3-furan-carboxylic acid (kojic acid) is as shown in **Figure 7**.

The results of the anticancer activity test of this compound isolate were carried out in vitro using HeLa cells using MTT Method. The results of the anticancer activity test are shown in **Table 3.** Based on the data, the anticancer activity test results of the compound isolates show that the IC_{50} value obtained is 413.695 ppm. Based on the test results, it turns out that isolate compounds in this category have weak cervical anticancer activity. Categories in cytotoxic compounds according to U.S. National Cancer Institute consist of 4 categories, namely the very toxic category if the IC_{50} value is ≤ 20 μg/mL, moderate cytotoxic category or quite active if the IC_{50} value is in the range of 21-200 μg/mL, weak cytotoxic category if the IC_{50} value is in the range 201-500 μg/mL and if the IC_{50} value is ≥ 500 μg/mL including non-toxic categories (Widiyastuti et al., 2019).

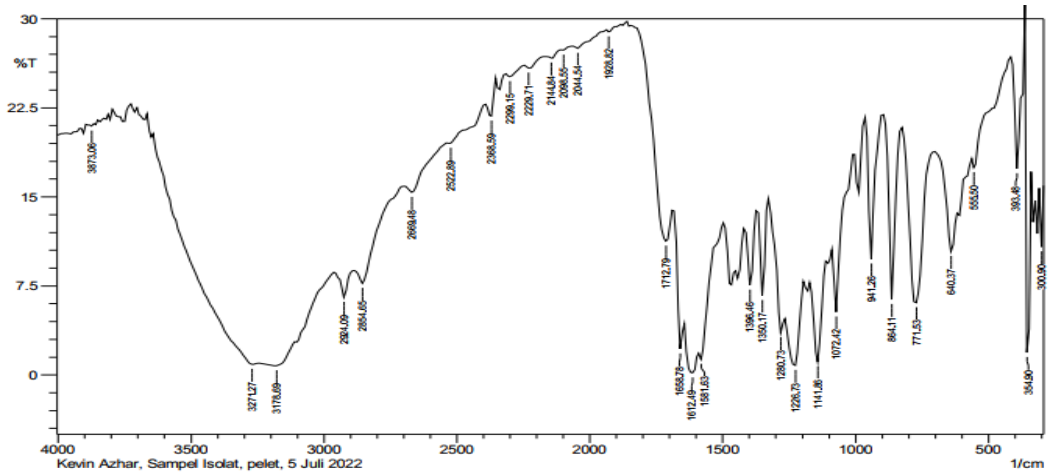

Figure 6. FTIR spectrum of isolate compound

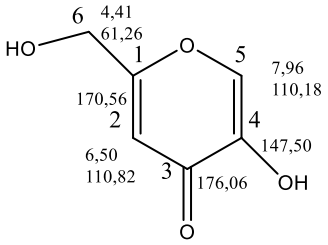

Figure 7. Molecular structure of the isolate compound which is kojic acid

Table 3. Data on cervical anticancer activity test results on pure samples isolate compounds against HeLa cells

Concentration	% Inhibition	IC_{50} (ppm)
31.25	7.59	
62.5	10.67	
125	19.64	**413.69**
250	39.48	
500	43.98	
1000	82.52	

The results of the anticancer activity test against HeLa cells from the ethyl acetate fraction before purification were IC_{50} amounting to 165.37 ppm and falls into the quite active category (Chasani et al., 2022). It is suspected that the anticancer activity occurs synergistically between the isolate compound (Kojic acid) and several other compounds (impurities) contained in the fraction, including compounds from the saponin and terpenoid compounds and several other compounds that were not detected in the color test as shown in **Figure 1**.

Kojic acid is a metabolic product of fungi including Aspergillus species. No literature has been found that explains the findings of kojic acid in plants. Therefore, the results of this study are new findings which have obtained compounds that are strongly suspected to be kojic acid derived from the ethyl acetate fraction of *T.catappa* fruit flesh. Kojic acid is a natural metabolite that is generally produced by Mushrooms have the ability to inhibit tyrosinase activity in melanin synthesis. The major applications of kojic acid and its derivatives in medicine are based on their biocompatibility, antimicrobial and antiviral, antitumor, antidiabetic, anticancer, anti-speck, anti-parasitic, and pesticidal and insecticidal properties (Saeedi et al., 2019). Ola, et al., 2019, found kojic acid in a train of endophytic fungi, namely the endophytic fungus A. flavus from the stem of *Catharanthus roseus* and the fungus *A. flavus* from Annona squamosa and Curcuma xanthorisa (Ola et al., 2019). Kojic acid in the endophytic fungus *C. Gloeosporioides* and it has antimicrobial activity (Nurunnabi et al., 2018). Kojic acid isolated from Aspergillus tamarii MM11 showed good cytotoxic activity on HepG-2 cell line. These data indicate that kojic acid has potential as an antitumor agent (El-Metwally et al., 2020). Kojic acid derivative compounds are reported to have potential as anti-cancer agents (Karakaya et al., 2019). Kojic acid and its derivatives cause cytotoxicity in several cancer cell lines, including melanoma, hepatocellular carcinoma, ovarian cancer, breast cancer, and colon cancer (Zilles et al., 2022). Kojic acid or its derivatives are relatively safe for humans in the concentrations tested. Therefore, the application of kojic acid and its

derivatives in cosmetic, pharmaceutical or medical fields is very hopeful (Zilles et al., 2022). In the cosmetics industry, Kojic acid and Its derivatives are used as anti-oxidants, anti-proliferative, anti-inflammatory, radioprotective and skin brightening agents, agents in skin creams, lotions, soaps, and dental care products. KA has the ability to protect against UV rays, suppressor of hyperpigmentation in humans and inhibitor of melanin formation, due to inhibition of tyrosinase activity (Saeedi et al., 2019). The Kojic acid in *T. catappa* Linn fruit flesh that researchers found provides information on the potential of *T. catappa* fruit flesh extract as an additive in cosmetics, especially for various products related to skin health and for skin care. Widyaningsih et al. 2018 have obtained research results on antioxidant liquid soap with the addition of ethyl acetate fraction of T. catappa Linn fruit pulp as an active ingredient. The resulting liquid soap product liquid soap product that had good characteristics with an antioxidant value, IC_{50} = 79.51 ppm (Widyaningsih et al., 2018)

CONCLUSIONS

The compound isolate purified from the ethyl acetate fraction of *T. catappa* Linn fruit flesh is clear yellowish crystals, has a melting point of 150 ± 2°C and has anticancer activity against HeLa cells IC_{50} = 413.695 ppm. The compound isolate is strongly suspected to be the compound 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (Kojic acid) with a molecular weight of 142.36 gram/mol. The results of this study provide information on the potential of *T. catappa* fruit flesh extract as an additive in cosmetics, especially for various products related to skin health and for skin care and also to be developed as a medicinal material.

ACKNOWLEDGEMENTS

All of this research was funded by Universitas Jenderal Soedirman through Unsoed BLU 2022 Fundamental Research funding.

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