Isolation flavonoid compound chrysoeriol from ethyl acetate extract of zaitun leaves (*Olea europaea*)

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Abstract. The Olive Tree (*Olea europaea*) is a plant originating in the Middle East. Olea Europaea plant is contained several secondary metabolites such as olive oil, phenolic compounds, flavonoids, triterpen, biphenol. The aims of this research are to isolate and identify a flavonoid compound that extracted in the ethyl acetate of olive leaves. Isolation was carried out by meseration, liquid vacuum chromatography, column chromatography and thin layer chromatography. Isolation results obtained 10 mg isolates were identified using proton nuclear magnetic spectroscopy (1H-NMR) and carbon (13C-NMR). According to spectra analysis, describe that isolate is chrysoeriol.

1. Introduction

Indonesia is tropical country where almost already all plants can grow well. One of them is olive tree as a plant came from Mediterian or Middle East countries. It can be cultivated in Padasuka Bandung [1].

The Olive plants contains several useful compounds such as mineral, primary and secondary metabolites [2]. The main primary metabolites is oil which obtained from seeds. The secondary metabolites covered terpenoids, alkaloids, flavonoids, saponins, tanins, steroids [3].

Some influencing factors can affect production of secondary metabolites such as cultivated, nutrients, environmental factors, pest, light intensity, pesticides, soil fertility, humidity and water supply [4].

Olive plants are widely used as medicine. Dried leaves and fruit are used to treat diarrhea, respiratory and urinary tract infections, stomach and intestinal ailments, and as a mouth cleaner. Hot water extract of olive leaf is taken to treat high blood pressure and also fruit essential oil extract is taken to treat kidney [5].

Researchers are developing how to isolate 1,1-diphenyl-2-picrylhidrazine compounds and its derivate as one component of compounds in olive plants that have high antioxidants [6]. Based on this, it is necessary to find out what groups of secondary metabolites contained in the ethyl acetate extract of *Olea europaea* leaves, isolation and structure elucidate.

2. Materials and methods

2.1. Materials

Fractionated distillation apparatus, blender, macerator, bottle vial 10 mL, all kind of lab glassware, Buchi rotary evaporator, silica gel GF60, silica gel TLC plate 60 F254 (E. Merck 05554), several size of
columns chromatography, ultraviolet lamp 245 nm and 366 nm wavelength, pipete, aluminium foil, aquadest, methanol, ethyl acetate, n-hexane, chloroform p.a (Merck), Dragendorff reagent, a plate of Mg, ethanol p.a, acetate anhidrids p.a (Merck), H$_2$SO$_4$ p.a (Merck), HCl 2N, FeCl$_3$ 1%.

2.2. **Instrument**
NMR Agilent 500 MHz with DD2 console system which frequency operated in 500 MHz ($^1$H) and 125 MHz ($^{13}$C) used (CD$_3$)$_2$CO solvent.

3. **Methods**

3.1. **Extraction**
750 g fresh leaves are washed and dried at room temperature (avoid direct sunlight). The leaves are crushed using a blender. Coarse leaf samples were extracted using maceration method using ethyl acetate solvent in the macerator for 3 days. The solvent is replaced every 1 × 24 hours with a new solvent. Maceration solutions are evaporated using a rotary evaporator with a temperature of 40 ℃. Concentrated extracts (I, II, III) combined to produce 34 grams of concentrated extracts.

3.2. **Alkaloids test**
The sample is added 1-2 drops of Dragendorff reagent and observed changes occur. Alkaloid positive test is characterized by the formation of orange deposits.

3.3. **Flavonoids test**
Samples were added with a little of Mg powder, then add 2 ml of a solution of EtOH: HCl (1: 1) and 5 ml of amyl alcohol, shake the mixed solution and observe the changes that occur. The flavonoid positive test on the sample is characterized by a change in the color of the solution to red, yellow, or orange.

3.4. **Steroids/terpenoids test**
Samples are added 2 drops of acetic anhydride solution and stir, add 1-2 drops of concentrated H$_2$SO$_4$, then shake the solution slowly, allow a few minutes and observe the changes that occur. A positive steroid test is characterized by a change in the color of the solution to blue or green, while a positive terpenoid test gives a change in red or violet color.

3.5. **Saponins test**
Shake the sample in a test tube for 10 seconds, if formed 1-10 cm foam is stable for 10 minutes, then the sample contains saponin, then add 1 drop of 2N HCl solution. Test positive for saponin if foam is lost.

3.6. **Tanin test**
Samples are added 2-3 drops of 1% FeCl$_3$ solution and changes occur. The tannin positive test will show a change in the color of the bluish black or green solution.

3.7. **Isolation of flavonoids compound from ethyl acetate extract of olive leaves**
Concentrated extract was carried out by TLC using solvents with polarity (n-hexane: ethyl-acetate): (10: 0), (9: 1), (8: 2), (7: 3), (6: 4), (5: 5), (4: 6), (3: 7), (2: 8), (1: 9). The mobile phase to be used is an isocratic eluent. The selected isocratic effluent is a solvent with polarity of n-hexane: EtOAc (6: 4). Blemishes were seen using ultraviolet visible light at λ 254 nm and 366 nm. Plates that display colorless stains are sprayed using a universal solvent mixture of H$_2$SO$_4$ solution dissolved in ethanol by comparison (20:80).

Concentrated extract of 30 grams is fractionated with KVC. The sample was fractionated using eluent with polarity (n-hexane: EtOAc): (0); (8: 2); (7: 3) 2 times; (6: 4) 3 times; (5: 5) 2 times. Fractionation
produces nine fractions, this solution is then evaporated with a rotary evaporator at 40 °C, carried out by TLC.

Fractions 6-9 have the same chromatogram pattern, combined to produce a sample of 2 grams and then in the KKG with a mobile phase with a solvent (n-hexane: EtOAc) / (6: 4). The KKG results were produced 130 vials, each vial containing 10 ml of a sample, then randomized to TLC. Samples that have the same chromatogram pattern are combined, resulting fractions A, B, C, D, E and F.

F fraction was washed with solvents with higher polarity, namely n-hexane, chloroform, ethyl acetate and methanol. The sample of F ethyl acetate and methanol fraction as much as 40.10 mg was then carried out KKG again produced 10 mg isolates, then in TLC and flavonoid test.

3.8. Structure identification of isolate
Isolates of 10 mg were characterized by proton nuclear magnetic resonance spectroscopy (1H-NMR) and carbon nuclei (13C-NMR). The DD2 console system operates at frequencies of 500 MHz (1H) and 125 MHz (13C) with solvent (CD3) 2CO.

4. Results and discussion

4.1. Sample preparation
Olea Europaea plant seeds come from Arabic, Owner cultivate plants in Padasuka, Bandung, West Java. Olea europaea leaves are washed with running water so that dirt and pests attached to the leaves do not affect the results of isolation. The leaves are dried at room temperature to remove the water content of the leaves, during the drying process the leaves are avoided from direct sunlight so that the active compounds contained are not damaged.

Dry leaves are crushed using a blender to form small, coarse powders which aim to facilitate the withdrawal of active compounds during the extraction process. The smaller the sample size, the greater the surface area of the sample and the interaction of solvent contacts in extraction will be greater, so the extraction process will be more effective.

Olea eoropaea sample extraction uses the simplest method, namely maceration. Coarse samples are immersed using semi-polar ethyl acetate solvents to withdraw the content of semi-polar active compounds, when the meseration process occurs a dissolution process (the process of submerging the target compound by the solvent) and diffusion (the process of carrying compounds by the solvent to leave the cell or leaf matrix Rough). Maceration solution is evaporated with a temperature of 40 °C so that the content of active compounds that cannot stand high temperatures is not damaged.

4.2. Phytochemistry test

4.2.1. Flavonoids test. Concentrated extract added with a solution of EtOH: HCl hydrolysis reaction occurs flavonoid compounds into aglycones, namely hydrolyzing O-glycosyl. Mg powder plays a role in reducing flavonoid compounds. Amyl alcohol is used to dissolve flavonoids, the solution changes color to yellow indicating that positive samples contain flavonoid chemicals.

4.2.2. Steroids test. Concentrated extract added with acetic anhydride solution and concentrated H2SO4 solution oxidation reaction occurs in the group of terpenoid / steroid compounds. When concentrated H2SO4 is added to the sample, the acetic anhydride reacts with acids so that the C atoms in the acetic anhydride form a carbocation. The carbocation formed reacts with the O atom in the -OH group present in the triterpenoid compound. The reaction that takes place is the esterification reaction, namely the formation of ester compounds by triterpenoid compounds with anhydrous acid acetic acid. Concentrated positive extract containing steroids is characterized by a change in the color of the solution to green.

4.2.3. Tanins test. Concentrated extract added with 1% FeCl3 solution hydrolysis reaction occurs in the tannins so that the green color changes to blackish, tannins react with FeCl3 to form complexes of Fe3
+ −-tannin and Fe3 + − polyphenols. Oxygen atoms in tannins and polyphenols have electron pairs capable of donating electrons to Fe3 + which have vacant d orbitals forming coordinate covalent bonds to form complex compounds. Positive samples contain chemical compounds of tannins [7].

4.3. Isolation of flavonoid compounds from olive leaf ethyl acetate extract

The isolation process was carried out using the KVC, KKG and KLT methods. In the research used isocratic eluent, which is the mobile phase used has a solvent composition that does not change / remain. TLC testing uses solvents with polarity (n-hexane: ethyl-acetate): (10: 0), (9: 1), (8: 2), (7: 3), (6: 4), (5: 5), (4: 6), (3: 7), (2: 8), (1: 9) is to determine the isolated target compound and determine the isocratic eluent suitable for the isolation process, the results of the chromatogram obtained in Figure 1.

Figure 1. TLC of target compound.

The isolated target compound has a Rf value of 0.32 and the isocratic eluent used is eluent with polarity of n-hexane: ethyl-acetate (6: 4). Crude samples are fractionated by the KVC technique to separate chemical compounds in crude extracts to be simplified based on their polarity.

In the KVC step a single / pure compound is not directly produced, therefore further separation is required. The next separation was that the KKG produced 6 fractions (A-F). F fraction is in the form of smooth yellow solid. F fraction has two distant stains, fraction F is washed with a low to high polarity solvent for the separation of the target compound. F ethyl acetate and metaol fractions on the chromatogram still have 2 distant stains, then the KKG was re-done to produce 10 mg of isolates.

The isolates were then subjected to phytochemical tests to obtain positive results of flavonoids characterized by a change in the color of the solution to red. TLC test results obtained chromatogram Figure 2, isolates already have one stain.

Figure 2. TLC of isolate.
The isolate has an Rf value of 0.35, the isolate stains appear on the chromatogram seen visibly in yellow, and the isolate stains appear to be complementary to blue when viewed under UV light.

4.4. Structure identification of isolate

The characterization of Olea europaea isolates using NMR instruments obtained 1H-NMR spectrum and 13-NMR to predict the flavonoid compound of Olea europaea isolates. The characterization of Olea Europaea isolates using 1H-NMR spectroscopy provides chemical shift information from 0-13 ppm which shows various types of protons, multiplicity, coupling constant (J) and number of protons.

Figure 3 shows the 1H-NMR spectrum of the isolate, in the picture there is a proton signal. The 1H-NMR spectrum shows that there is an aromatic proton ring of rings A and B at a chemical shift of 6.00-8.00 ppm. The chemical shift singlet signal 13.00 ppm has 1 proton (OH-5). Double doublet signal with chemical shift 7.92-7.95 ppm and coupling constant = 3.05 Hz and 2.1 Hz shows proton H-2'. Triple doublet signal with chemical shift 7.59-7.63 ppm and constant coupling = 2.15 Hz, 2.15 Hz, 2.2 Hz denotes the H-6 proton. Triple signal with a chemical shift of 7.00-7.03 ppm as H-5'.

Doublet signal with chemical shift 6.69-6.70 ppm and coupling constant = 1.95 Hz as proton H-8. The doublet signal with a chemical shift of 6.634-6.639 ppm and the coupling constant = 1.85 Hz indicates the proton H-3. The double doublet signal with a chemical shift of 6.53-6.54 ppm and the coupling constant = 2.25 Hz and 2.2 Hz are identified as H-6.

Singlet signals with a chemical shift of 4.00 ppm have 3 protons are (~OCH3) methyl groups, the three methoxy protons are chemical equivalents, there are no neighboring protons so there is no signal breakdown and appear as singlet signals. The coupling constant is the distance between two signals in a doublet. Proton H-3, H-6, H-8, H-2', H-5' and H-6' are methyl protons.

13-C NMR for the purpose of structural elucidation of organic compounds is very important, because it can provide information about the amount of carbon signals in organic compounds, carbon signal breakdown that depends on the number of hydrogen atoms bound (metin, methylene, methyl and quaternary carbon), carbon types (sp, sp2, sp3 and carbon quartener) as well as electronic environments that affect the chemical shift of each carbon atom in the molecules of organic compounds [8].

Chemical shift of sp3 carbon-carbon when there is no electronegative atom is around 0-60 ppm, for sp2 carbon it is generally shifted chemically between 100-160 ppm, for aldehydes and ketones between 175-200 ppm, and for chemical shifts of carbon sp or alkyne between 63-95 ppm. The 13C-NMR spectrum of the isolate can be seen in Figure 4.
Figure 4 shows the $^{13}$C-NMR spectrum of the isolate, identified that the isolate has one visible carbonyl carbon signal at the normal low field position of 178.8 ppm. Six carbon sp atom (metin) at 129.2 ppm, 126.2 ppm, 116.8 ppm, 104.1 ppm, 99.7 ppm, 94.7 ppm. Six oxygenated aromatic signals appear at the field position of 164.8 ppm, 163.3 ppm, 161.9 ppm, 158.7 ppm, 145 ppm and 116.8 ppm. Two non-oxygenated aromatic carbon signals appear at 121.3 ppm and 104.4 ppm. One sp3 (methyl) carbon signal appears at the field position of 56.1 ppm.

Based on this identification, the amount of carbon contained in isolates was 16, there were hydroxyl groups on carbon C-5, C-7 and C-4. The methoxy function group is found in carbon C-3. The correlation between $\delta$C and $\sum$H, $\delta$H (ppm), interpretation, and the coupling constant (Hz) shows that the isolate has 16 carbon amounts, 12 protons and 12 O.

The isolated isolate of ethyl acetate olive leaf has the molecular formula C16H12O6. This compound is identified as chrysoeriol and has the name IUPAC 5,7-dihydroxy-2-(4-hydroxy-3-methoxy phenyl) chromen-4-one.

5. Conclusion
Groups of secondary metabolites contained in the ethyl acetate fraction of Olea europaea leaves are flavonoids, steroids and tannins. Identification of the structure of the compound flavonoid isolates of the olive acetate fraction ethyl acetate is Chrysoeriol.
Table 1. Comparative Spectrum $^{13}$C-NMR and $^1$H-NMR between Isolate and reference.

| No. | Reference (Aseton-d$_6$) [9] | Isolate (Aseton-d$_6$) |
|-----|-----------------------------|------------------------|
|     | $^{13}$C-NMR | $^1$H-NMR | $^{13}$C-NMR | $^1$H-NMR |
| 2   | 164.9           | -          | 163.3        | -          |
| 3   | 104.5           | 6.69 (s)   | 104.1        | 6.63 (d, 1.85 Hz) |
| 4   | 182.9           | -          | 178.8        | -          |
| 5   | 163.3           | -          | 161.9        | -          |
| 6   | 99.7            | 6.26 (d, 2.0 Hz) | 99.7        | 6.53 (dd, 2.25 Hz, 2.2 Hz) |
| 7   | 165.0           | -          | 164.8        | -          |
| 8   | 94.7            | 6.55 (d, 2.0 Hz) | 94.7        | 6.69 (d, 1.95 Hz) |
| 9   | 157.8           | -          | 158.7        | -          |
| 10  | 152.2           | -          | 152.2        | -          |
| 1’  | 123.6           | -          | 121.3        | -          |
| 2’  | 110.7           | 7.63 (d, 2.1 Hz) | 129.2       | 7.92 (dd, 3.05 Hz, 2.1 Hz) |
| 3’  | 148.8           | -          | 116.8        | -          |
| 4’  | 151.3           | -          | 145.0        | -          |
| 5’  | 116.4           | 7.01(d, 8.3 Hz) | 116.3       | 7.00 (t) |
| 6’  | 121.4           | 7.60 (dd, 8.3 Hz, 2.1 Hz) | 126.2       | 7.59 (td, 2.15 Hz, 2.15 Hz, 2.2 Hz) |
| 1”  | 56.5            | 4.00 (s)   | 56.1         | 4.00 (s)  |

where: $s$ = singlet; $m$ = multiplet; $d$ = doublet; $dd$ = double doublet; $td$ = triple doublet; $tdd$ = triple double doublet; $q$ = quartet.

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