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ISOLATION OF ACTIVE COMPOUND FROM RED POMEGRANATE *(PUNICA GRANATUM L.)* SEEDS ETHANOLIC EXTRACTS

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ABSTRACT

Objective: Pomegranate (*Punica granatum L.*) is rich in antioxidants and antibacterials, lending itself to beneficial effects on health improvement. The seeds contain a variety of active compounds. The purpose of this research was to isolate the active compound of red pomegranate seeds through ethanolic extraction.

Methods: Pomegranate seeds were extracted into 24 hexane fractions of the concentrated crude extract and left in open vials until all the solvent evaporated, leaving behind crystals. Crystals in vials 11, 12, and 13 were purified using n-hexane, ethyl acetate, methanol, chloroform, acetone and isopropyl alcohol as the solvents. The crystals were monitored with Thin Layer Chromatography (TLC) on a Kiesel gel 254 GF plate (e. Merck) using the developing system of hexane: ethyl acetate: formic acid (1:1:0.1) mixture and a UV light spot viewer with 366 wavelength emissions. Purification test with TLC two directions using hexane: ethyl acetate: formic acid (1:1:0.1) mixture as the eluents for the first development, continued with the second development using chloroform: methanol: acetate acid (9:0.5:0.5) mixture. The Retention Factor (Rf) value for the spot was measured.

Results: Chromatogram showed only 1 spot with Rf value 0.88.

Conclusion: There is one pure (active) isolate in red pomegranate seeds ethanolic extract.

Keywords: Active compound, Ethanolic extracts, Fraction, Isolation, Red pomegranate seeds

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INTRODUCTION

Pomegranate (*Punica granatum L.*), one of the fruit plants referred to as "nature's power fruit" due to its health-promoting effects. This fruit has antioxidant activity due to the abundance of compounds such as polyphenols, flavonoids, flavones, anthocyanins and catechins located in different parts including fruits, seeds, and peels, It is known to have antibacterial properties too [1-6].

The pomegranate fruit is often used in several systems of medicine for a variety of ailments as traditional medicine, but it is not yet known the active substances contained in the seeds; therefore the use of this plant still needs to be studied to determine the active compound. The extract was obtained through the maceration process as a crude extract. Crude extracts still contain a mixture of several compounds that needs to be further purified through a fractionation process [7].

Fractionation using chromatography is the separation of the main content from the other main contents based on the level of the polarity depends on the type of compounds contained in the plant or of two or more compounds or ions by the distribution between two phases, one which is moving and the other which is stationary [7-10].

Liquid-liquid extraction (multilevel extraction) generally begins with a less polar solvent and continued with a more polar solvent. The degree of polarity can be determined from the values of the dielectric constant of the solvent. The four phases of the liquid-liquid extraction using four kinds of solvent were extraction of acetone, n-hexane, ethyl ether and ethyl acetate. Stages of fractionation, separation, and purification can be done in various techniques including chromatography methods or combinations of chromatography with other methods. The separation and purification of plant constituents are mainly carried out using one or other, or a combination, of four chromatography techniques. The four techniques of chromatography are paper chromatography (PC), thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and high-performance liquid chromatography (HPLC) [7, 11, 12].

TLC is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC is a quick, inexpensive microscale technique that can be used to determine the number of components in a mixture, verify a substance's identity, monitor the progress of a reaction, determine appropriate conditions for column chromatography, analyze the fractions obtained from column chromatography [8, 11, 13].

Fractionation of pomegranate active compounds has already been done. Growther *et al.* researched the pomegranate peel extract using methanol as the solvent in the maceration process. The result showed that the methanol extract of pomegranate peels contains antibacterial compounds against E. coli and TLC indicates the presence of ellagic acid [14]. A previous study was conducted by Setiadhi *et al.* on red pomegranate seed extract using ethanol as the solvent during the maceration process, found that column chromatography against hexane fraction of red pomegranate seeds ethanolic extract obtained 24 fractions and the TLC chromatogram of those fractions assumed there were 4 groups of compounds according to the visible spots i. e a thick spot in the fraction 6 while fractions 5, 7, and 8 contained aligned spots, as did 9-11 and 12-14, but the active compounds were unknown and therefore a further study should be done.

During thin layer chromatography, spots of a chemical mixture are placed on a flat plate coated with a layer of absorbent material and the lower portion of the plate is submerged insolvent. As the solvent moves up the plate, chemicals in the mixture move up with it at different rates causing separation. After the separation is complete, individual compounds appear as spots separated vertically [12, 15].

Each spot has a retention factor (Rf) value, that is defined as the distance a compound moves in chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, divided by the distance between the origin and the solvent front *(i.e.* the distance the solvent travels). It can be calculated using the formula [12, 15, 16]:

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

This value lies between 0.01 and 0.99 [7]. The larger an Rf of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger Rf is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. A compound of low polarity will have a larger Rf value than a polar compound run on the same plate. The Rf can provide corroborative evidence as to the identity of a compound. If two substances have the same Rf value, they are likely (but not necessarily) the same compound but if they have different Rf values, they are definitely different compounds [12].

The purpose of this research was to isolate the active compound of red pomegranate seeds ethanolic extract and to find out the Rf value of the active compound.

MATERIALS AND METHODS

Material

A laboratory experiment was conducted using red pomegranate seeds ethanolic extract at the Laboratory of Pusat Antar Universitas, Bandung Institute of Technology.

Preparation of crude extract

The red pomegranates were collected from Sindang Anom Garut, West Java, Indonesia. It was peeled off, blended, filtered, dried for 2 x 24 h at 48 °C and mashed into powder. The powder was extracted with 96% ethanol and the extract was concentrated using a rotary evaporator until the concentrated crude extract was obtained.

Fractionation by column chromatography and TLC techniques

The concentrated crude extract was purified by column chromatography using hexane as a solvent to obtain 24 vials, numbered in sequence. The obtained 24 fractions were monitored by TLC. After the spots were dry, the plate was put into a chamber that was filled with a mobile phase that will move up to the end of development. The plate was removed from the chamber and observed under the 254 nm light. These tests have been done in the earlier studies. All solvents evaporated in open air and crystals were left behind in the vials.

Crystal's purification

The crystals in the 11, 12 and 13 fractions were washed with solvents that could dissolve pollutants but does not dissolve the crystals. These solvents were n-hexane, ethyl acetate, methanol, chloroform, acetone and isopropyl alcohol [17].

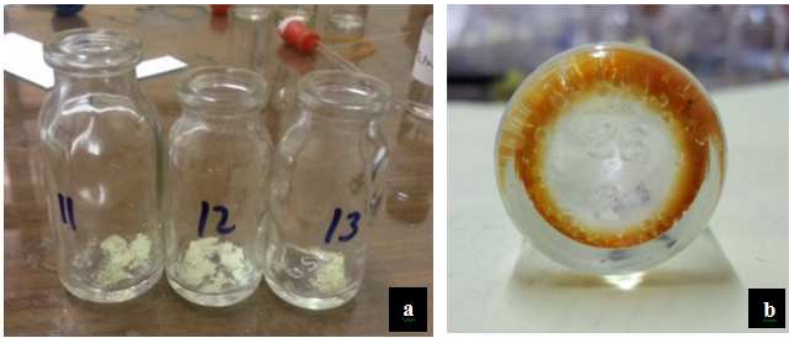

Fig. 1: Crystal purification. (a). Crystals infractions no 11, 12 and 13. (b). Crystals mixed with the pollutant

Crystals mixed with pollutant in vial 13 after the solvent evaporated fig. 1b, were washed until the pollutant dissolved but the crystals did not dissolve and left them to precipitate.

Thin-layer chromatography

The crystals were monitored with TLC on a Kiesel gel 254 GF plate (e. Merck) using the developing system of hexane: ethyl acetate: formic acid (1:1:0.1) mixture and a UV light spot viewer with 366 wavelength emissions and the spot was identified.

To make sure that there is only 1 active compound, purification test with TLC 2 directions was done using hexane: ethyl acetate: formic acid (1:1:0.1) mixture as the eluents for the first development, continued with the second development using chloroform: methanol: acetate acid (9:0.5:0.5) mixture as the eluents. The Rf value for the spot was measured [16]

RESULTS AND DISCUSSION

Chromatogram of the crystal in vial 12 showed there were 2 spots fig. 2a and the washed crystals in vial13 produced dry crystals fig. 2b.

Chromatogram of TLC from these dry crystals on a Kiesel gel 254 GF plate (e. Merck) with the developing system of hexane: ethyl acetate: formic acid (1:1:0.1) mixture and a UV light spot viewer with 366 wavelength emissions showed 1 spot with Rf value 0.88 fig. 3a which was predicted as the active compound of red pomegranate.

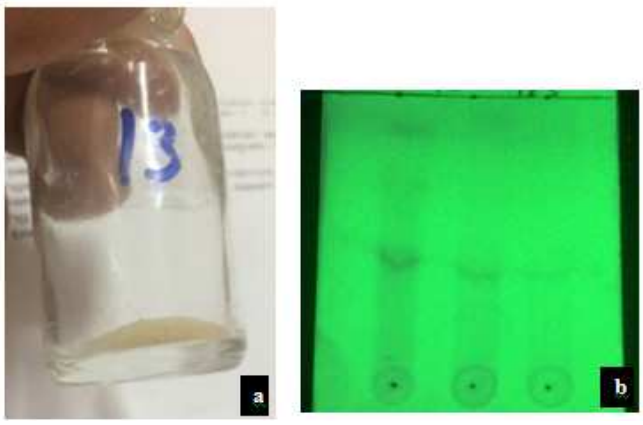

Fig. 2: Crystals. (a) Chromatogram of crystals in vial 12. (b) Dry crystals in vial 13

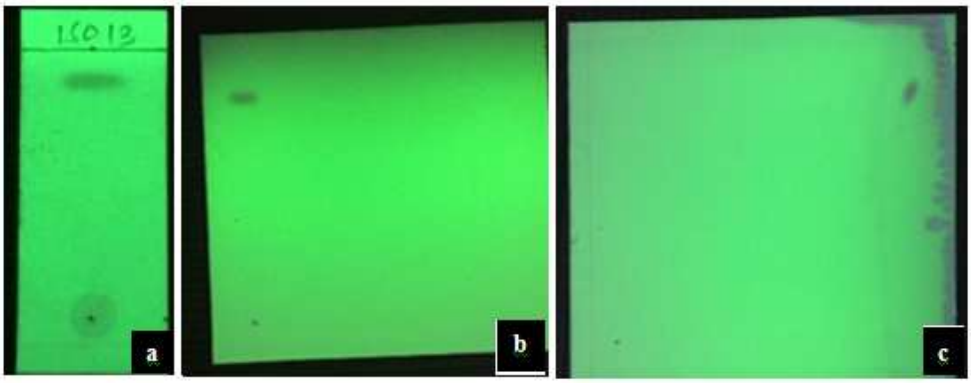

Fig. 3: Chromatogram of crystals in vial 13: (a) 1 spot Rf value 0.88. (b) On the first development. (c) On the second development

Purification test with TLC 2 directions fig. 3a and 3b were done to make sure that there was only 1 active compound. The result showed that there was only 1 spot with Rf value 0.88.

The elusion against hexane fraction through chromatography column obtained 24 fractions which were then monitored with TLC. Chromatogram results showed 1 thick spot in the 6th fraction, similar spots in fractions 5, 7, 8, at fractions 9-11 and at fractions 12,-14. As there was a thick spot in the 6th fraction, it was assumed that in the fraction contained the active compound, but after the solvents evaporated there were only a few crystals while fractions 11-13 contained a lot of crystals. Fraction 6 showed a thick spot because it contained a lot of pollutants, while fractions 11-13 showed only thin spots on the TLC but after the solvents evaporated there were a lot of crystals. It means that they contained crystals and only a few pollutants, therefore, the study focused on fractions 11-13 because of the possibility that the crystals were the active compound of the red pomegranate seeds. The crystals were washed to purify them using several solvents that could dissolve the pollutants but does not dissolve the crystals while the other solvents could dissolve neither the pollutant nor the crystals.

Chromatogram of TLC from vial 12 was performed on a Kiesel gel 254 GF plate (e. Merck) with the developing system of hexane: ethyl acetate: formic acid (1:1:0.1) mixture and a UV light spot viewer with 366 nm wavelength emissions. There were 2 spots visualized. These spots indicated that the sample was not pure and must be purified again.

After the solvent in vial 13 evaporated/dried, there were crystals mixed with the pollutant. Crystals and pollutant were washed until they were separated and the crystals were left to dry. Chromatogram of TLC from dry crystals in vial 13 showed 1 spot with Rf value 0.88 [16], predicted as the active compound of red pomegranate.

To make sure that there was only 1 active compound, a purification test with TLC in 2 directions was done and the result showed that there was only 1 round spot with the same Rf value at 0.88 [16].

The Rf value can be used to the qualitative identity of an unknown compound compare with the standard compound. If the Rf value is the same, it means that both compounds are identical [15, 16]. TLC 1 and 2 directions against crystals in vial 13 obtained 1 round spot with the same Rf value i. e 0.88, it showed that the isolated compound is the active compound of red pomegranate seeds.

The finding of this study is useful for our further research i. e determining the chemical structure and minimum inhibitory concentration (MIC) as well as minimum bactericide concentration (MBC) of the active compound of red pomegranate seeds against bacteria Streptococcus sanguis as the cause of recurrent aphthous stomatitis.

CONCLUSION

The present study showed that the active compound in the red pomegranate seeds has an Rf value of 0.88.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

No conflict of interest

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