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Bioassay-guided isolation of the antioxidant constituent from *Kaempferia rotunda* L.

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Abstract. *Aryantini D, Astuti P, Yuniarti N, Wahyuono S. 2023. Bioassay-guided isolation of the antioxidant constituent from* Kaempferia rotunda *L. Biodiversitas 24: 3641-3647. Kaempferia rotunda L.* contains various phytochemical compounds with various biological activities and has been widely utilized in traditional medicine. This research focused on exploring bioactive compounds in the ethanol extract of *Kaempferia rotunda* using radical scavenging (1,1-Diphenyl-2-picrylhydrazyl) bioassay-guided isolation. Initially, the powdered material of *K. rotunda* was macerated with 70% ethanol and filtered, and the filtrate was evaporated in vacuo to make concentrated ethanol extract. The concentrated ethanol extract was then triturated gradually with increasing polarity of solvents (n-hexane, ethyl acetate, ethanol) to give n-hexane (HSF), ethyl acetate (EASF), ethanol (ESF), and residue (ISF) fractions. Each fraction was tested by DPPH bioassay with quercetin as the positive control to determine the active fraction. The active fraction (ESF) was further fractionated by Vacum Liquid Chromatography (VLC) using a mobile gradient phase starting from ethyl acetate 100%, acetone 100%, and methanol 100% to make 3 sub-fractions (F1, F2, F3), respectively. The DPPH radical scavenging bioassay showed that F1 was the most active, containing bioactive compound detected by TLC visualized by DPPH. Based on spectroscopic and literature data comparison, this compound was isolated, purified, and identified as crotepoxide. Crotepoxide displayed IC_{50} of 38.91±0.59 (ABTS), 47.45±0.60 (DPPH), and 26.74±1.23 (FRAP) µg/mL.

Keywords: Chromatographic techniques, crotepoxide, DPPH bio-guided isolation, *Kaempferia rotunda*

INTRODUCTION

Oxidative stress is a situation that manifests itself when there is an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues. The capacity of biological systems will detoxify these reactive products. This imbalance can lead to oxidative stress, resulting in oxidative damage (Pizzino et al. 2017). Damage caused by oxidative stress can occur at any level, from the level of cells and tissues to the level of organs. Oxygen free radicals, also known as Reactive Oxygen Species (ROS), are produced as a by-product of aerobic cellular metabolism. Several different stress situations exacerbate the formation of oxygen-free radicals. The presence of free radicals, which originated from various places, has led to the evolution of numerous defensive systems in living things. When the production of free radicals dominates endogenous antioxidants, biologically exogenous antioxidants are required. Exogenous antioxidants should be prospective to prevent side effects caused by exposure to ROS (Abiodun et al. 2020). Similarly, antioxidant chemicals participate in several stages of the defense mechanism, including prevention, radical capture, repair, and adaptability (Mucha et al. 2021).

Bioactive compounds derived from plants have been widely used as anticancer, anti-inflammatory, antitumor, antioxidant, and antimicrobial agents due to their pharmacological effects and minimal side effects. The development of natural antioxidants is a major area of research interest. Active chemicals found in plants create defense mechanisms through antioxidant systems; these systems work by converting free radicals into less reactive species. Natural antioxidants are essential in preventing or treating human diseases and interrupting any adversity that disrupts normal human health and is considered safe (Ramana et al. 2018). The antioxidant constituents in plants include catechin, kaempferol, quercetin, gallic acid, caffeic acid, and α-tocopherol are among the examples of phytochemical compounds that have been reported to exert good antioxidant (Yeap et al. 2017).

Kaempferia is a genus member of the Zingiberaceae family, which consists of approximately 60 species of rhizomatous herbs (Yeap et al. 2017). Various types of secondary metabolites with pharmacological activity of *Kaempferia* species have been reported, including the diterpenoid isopimarane, abietane, labdane, and clerodane diterpenoids, flavonoids, phenolic acids, phenyl-heptanoids, curcuminoids, tetrahydropyrano-phenolic, and steroids. In addition, several compounds such as kaempfolienol 2'-hydroxy-4,4',6'-trimethoxychalcone

isolated from *Kaempferia* species are potential antioxidants through ABTS, DPPH, CUPRAC and FRAP assays (Elshamy et al. 2019).

Kaempferia rotunda L. is a plant that is used in the rhizome part for the treatment of diarrhea, stomach ache, colic, and liquefied phlegm, eliminates vaginal discharge, tightens female muscle, inflammation of wounds, and bruises (Elshamy et al. 2019), as a slimming ingredient in herbal extracts (Rusiliati et al. 2003; Mudjiono et al. 2014). The first stage in determining the phytochemical makeup of medicinal plants is extraction. Extraction and solvent procedures significantly separate the bioactive elements from the rhizome of *K. rotunda*. Ethanol is the solvent chosen for extraction, capable of extracting a wide variety of different classes of compounds (Aryantini et al. 2022). Bioactive compounds and pharmacological activities that have been isolated from these plants include antimutagenic compounds 5-hydroxy-7-methoxyflavanone, 7-hydroxy-5-methoxyflavanone, and 5,7-dihydroxy flavanone (Atun et al. 2013; Atun 2014), as well as antioxidant compounds 2'-hydroxy'-4,4', 6'-tri methoxy-chalcone and crotepoxide (inactive, IC_{50} >1000 µg/mL (Lotulung et al. 2008). Nine compounds of antifeedant bioactive on *Spodoptera littoralis* larvae were also isolated from methanol extract of *K. rotunda* rhizome (Stevenson et al. 2007), which consists of a class of oxygenated cyclohexane compounds, acylation derivatives of 1-benzoyloxymethyl-1,6-epoxycyclohexan-2,3,4,5-tetrol, and salicin derivatives.

Although it has been reported that *K. rotunda* extract with various solvents performs antioxidant activity (Mohanty et al. 2008; Panyakaew et al. 2020), the main constituents in *K. rotunda* have not been studied extensively. Therefore, this study was conducted to isolate the active compound responsible for scavenging ABTS, DPPH radicals, and reducing capacity through a compound isolation method guided by in vitro bioassay.

MATERIALS AND METHODS

Plant materials

Kunci pepet (*Kaempferia rotunda* L.) was the study material that was received in November 2019 from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Tawangmangu, Karanganyar, Central Java. The simplicia used in the study was the rhizome of the *kunci pepet*. Plant determination was carried out at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. A voucher specimen (number: YK.01.03/2/677) was stored in the pharmaceutical biology laboratory of the Faculty of Pharmacy, Institut Ilmu Kesehatan Bhakti Wiyata Kediri.

Chemicals and equipment

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2-Azinobis [3-ethyl-benzothiazoline-6-sulfonic acid] (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ) and quercetin were purchased from Sigma Aldrich. Analytical grade solvents included ethanol, methanol, ethyl acetate (EA), chloroform, and n-hexane. Hydrochloric acid (HCl), Ferric (III) chloride hexahydrate ($FeCl_3.6H_2O$), potassium persulfate ($K_2S_2O_8$), Cerium (IV) sulfate tetrahydrate (Merck), silica gel F254, and silica Gel For PTLC F254 from Merck. The ultraviolet spectrum (methanol) was obtained from a UV spectrophotometer (Thermo Scientific Genesys 180), and Mass spectra were obtained from GC-MS (Shimadzu). The infrared (ATR) spectrum was obtained from a spectrophotometer (Shimadzu). Spectra of ^{1}H-NMR and ^{13}C-NMR ($CDCl_3$) were measured using Jeol JNM-ECS400 at 400 MHz.

Separation method

A total of 15 kg of simplicia was pulverized and sieved using a 30-mesh sieve. *K. rotunda* powder (12 kg) was macerated with 70% ethanol (1:7) in a vessel for 3x24 hours. Upon filtration, the filtrate was evaporated in a rotary evaporator to get a concentrated ethanol extract (KRE). The extract was stored in a tightly closed container, labeled, and stored in a desiccator.

KRE was then separated by trituration employing solvents gradually with increasing polarity of solvents [n-hexane, ethyl acetate, and ethanol] to make n-hexane (HSF), ethyl acetate (EASF), ethanol (ESF), and residue (ISF) fractions. All fractions were concentrated in a rotary evaporator (Buchi). The activity of the four fractions against DPPH radicals was tested by measuring the DPPH radical scavenging activity as guided by isolation Sudha and Srinivasan (2014). Vacuum Liquid Chromatography (VLC) further fractionated the active fraction to obtain 3 sub-fractions (F1-F3). The column [diameter of 8 cm filled with silica gel for TLC 60 (Merck)] and a fraction (dried with the stationary phase) was put on top of the stationary phase and eluted with the mobile phase in increasing polarity (ethyl acetate 100%, acetone 100%, and methanol 100%). VLC sub-fractions (F1-F3) were evaporated and measured for anti-radical activity (DPPH). The active fraction was subjected to isolating compound using preparative Thin Layer Chromatography (PTLC) with silica gel stationary phase for PTLC F_{254} 60 (Merck), eluted with chloroform: ethyl acetate (8:1 v/v). The isolated compound was then identified and characterized based on spectroscopic and reported literature data.

DPPH scavenging assay

The DPPH radical scavenging activity was measured using the modified Blois technique by Sudha and Srinivasan (2014). A concentration of 0.1 mM of DPPH was achieved by dissolving the extract/fraction/compound in methanol (pa). A series of extract concentrations (60, 80, 100, 120, and 160 µg/mL); fraction concentrations (40, 50, 60, 80, and 100 µg/mL); and compound (20, 25, 30, 40, and 50 g/mL) were incubated (in a dark room) for 30 minutes at 37°C with 50 mL of DPPH solution in methanol. The mixture was observed at a wavelength of 515.8 nm (Shimadzu UV 1780 spectrophotometer). All samples were measured in triplicate. The antioxidant inhibition (%) is calculated with the equation as follows in the formula:

$$\text{DPPH Scavenging Effect (\%)} = \frac{\text{abs. control} - \text{abs. sample}}{\text{abs. control}} \times 100\ \%$$

The IC_{50} value (concentration causing 50% inhibition) was determined by linearly graphing the extract/ fraction/compound concentrations against the relevant scavenging effect.

ABTS assay

ABTS was used to determine the free radical scavenging activity of the isolated compound according to the method reported previously (Rachkeeree et al. 2020) with minor modification. Briefly, the isolated compound was prepared by mixing 7.0 mM of ABTS, and 2.45 mM potassium persulfate ($K_2S_2O_8$) solutions were allowed to stand in the dark for 12 h at room temperature. The working solution was diluted to obtain a 0.7±0.05 unit absorbance at a wavelength of 734 nm. About 0.9 mL of diluted working solution was mixed with 100 µL of sample or standard. The free radical scavenging activity of the sample was measured by a spectrophotometer (Shimadzu) at a wavelength of 734 nm. All samples were measured in triplicate. The percentage inhibition of ABTS oxidation was calculated using the following formula :

$$\text{ABTS Scavenging Effect (\%)} = \frac{\text{abs. control} - \text{abs. sample}}{\text{abs. control}} \times 100\ \%$$

The concentration of compound or standard which exhibited 50% radical scavenging (IC_{50} value) was deduced from the linear regression of concentration versus the percentage inhibition.

FRAP assay

The reducing capacity of isolated compounds was investigated by applying the method reported previously with slight modification (Saravanan et al. 2023). Briefly, the FRAP reagent wash was freshly prepared by mixing 300 mM acetate buffer of pH 3.6, 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid (HCl), and 20 mM Ferric (III) chloride hexahydrate ($FeCl_3.6H_2O$). About 3 mL of FRAP reagent was mixed with a 100 µL sample or standard, and the absorbance readings were analyzed after 15 min at room temperature. The reducing capacity of the sample was measured by a spectrophotometer (Shimadzu) at a wavelength of 596 nm. All samples were measured in triplicate.

Calculation of the percentage of antioxidant activity can use the formula :

$$\text{FRAP Reducing Effect (100\%)} = \frac{\text{abs. control} - \text{abs. sample}}{abs.\ control} \times 100\ \%$$

Data analysis

The IC_{50} and inhibition (%) values were analyzed by one-way ANOVA with statistical significance $P<0.05$ using IBM ver. 25. The isolated compound was then identified and characterized using spectroscopic and reported literature data.

RESULTS AND DISCUSSION

Bioassay guided isolation of free radical scavenging agent

The concentrated ethanol extract of *K. rotunda* (KRE) was triturated gradually with increasing polarity of solvents [n-hexane, ethyl acetate, and ethanol] to make 4 fractions [HSF of 0.4334 g (2.167% w/w); EASF of 0.4934 g (2.467 % w/w); ESF of 4.62 g (23.1 % w/w), and ISF of 12.45 g (62.2 % w/w)]. This method is relatively easy to carry out and very effective and efficient because most samples can be separated simultaneously by separating compounds on a large scale. All fractions were–concentrated in a rotary evaporator (Buchi). Each step in the guided isolation bioassay was monitored by biological activity assay (Moyo et al. 2019); in this case, the 4 triturated fractions evaluated by the DPPH scavenging test, DPPH was used for its sensitivity, stable radical, simplicity of the user, and commercially available (Shah and Modi 2015; Suciyati et al. 2021).

The DPPH scavenging test measured the anti-radical activity of the four fractions by measuring the DPPH radical scavenging activity (Sharma et al. 2015) to obtain the active fraction, which would be proceeded to the separation stage with VLC. The activity of the four fractions against DPPH radicals was tested; the ESF turned out showing the strongest inhibition concentration compared to that of showed the strongest inhibition concentration (Figure 1) compared to the other fractions. The antioxidant activity was calculated as an IC_{50} value. The IC_{50} value was obtained from a linear regression equation plotting the inhibition percentage with the concentration (µg/mL).

The ESF fraction was the most active fraction with an inhibition concentration of 76.81±6.63 µg/mL (Figure 1). Further, it separated by VLC with ethyl acetate, acetone, and methanol as the mobile phase to obtain 3 sub-fractions [F1 (0.87 g), F2 (1.32 g), and F3 (2.0 g)]. The results of the DPPH test for F1-3 can be observed in Figure 1. The F1 is the selected fraction that actively possesses anti-radical DPPH with an Inhibition concentration of 77.98±1.17 µg/mL. F1 was then further separated by prep TLC with chloroform: ethyl acetate (8:1 v/v) as the mobile phase to make the isolated compound (Figure 2). Isolated compound obtained (182.9 mg) appeared as a colorless needle crystal having a melting point of 147-148°C.

Bioassay-guided isolation is a convenient procedure in which medicinal plant extracts are fractionated, and each fraction produced during the fractionation process is evaluated by bioassay Malviya and Malviya (2017). So that only the biologically active fraction is further isolated and purified (Pournaghi et al. 2021; Suharsanti et al. 2022). Bioassay-guided fractionation methods are used in new drug discovery because they directly link the extract analyzed and the target bioactive compound using a fractionation procedure followed by a specific biological activity. The KRE was quite active, which was possible because there were semi-polar and polar compounds soluble in the ethanol fraction, such as phenolic, non-phenolic, and tannins which contributed to DPPH scavenging activities (Wahyuningsih et al. 2021).

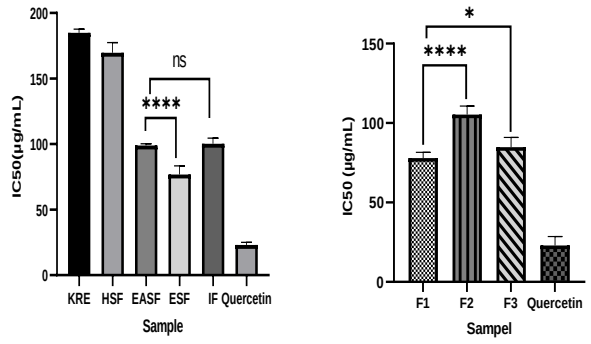

Figure 1. DPPH radical scavenging. A. Trituration fractions *Kaempferia rotunda* ethanolic extract (KRE), n-hexane fraction (HF), ethyl acetate fraction (EASF), ethanol fraction (ESF), an insoluble fraction (IF), and quercetin in IC50 (mg/mL). B. VLC sub-fractions ethyl acetate (F1), acetone (F2), methanol (F3), and quercetin in IC50 (mg/mL). Values are mean ±SD (n=3). * and **** is significant ($\alpha<0.05$) and ns: not significant ($\alpha>0.05$)

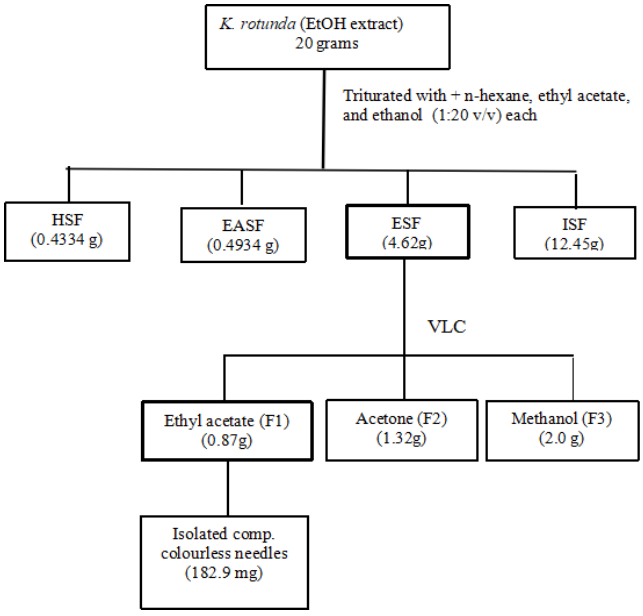

Figure 2. Isolation process of the isolated compound from *Kaempferia rotunda*

Therefore, the ESF is considered potentially contain anti-radical active compounds and then subjected for to further separation to obtain active compounds using VLC with increasing polarity (ethyl acetate, acetone, and methanol) to make sub-fraction 1 (F1), sub-fraction 2 (F2), and sub-fraction 3 (F3), respectively (Figure 1). The F1 was the strongest one, and then selected as the target for further separation and purification. According to the TLC profile, F1 still contains several compounds, which are then sprayed with 0.07 mM DPPH reagent to visualize the anti-radical active compounds. Most of the compounds in F1 showed absorption at 254 nm UV, which indicated the presence of unsaturated double bonds in the compound (Fadhilah et al. 2021) and shows that the compound having Rf 0.7 is the major compound that actively reduces DPPH and is also positive for $Ce(SO_4)_2$ reagent visualization. F1, as the active one, showing activity on TLC (visualized by DPPH, $UV_{254\ nm}$) was further subjected into isolation of compound using Preparative Thin Layer Chromatography (PTLC) with silica gel as stationary phase for PTLC F_{254} 60 (Merck). Compound observed at Rf 0.7 were then separated by preparative TLC (silica gel F_{254} for PTLC) and chloroform: ethyl acetate (8:1 v/v) as the mobile phase. The isolated compound appears as a single spot on TLC as observed by different solvent systems, this compound appears as colorless needle crystals (182.9 mg).

Compound characterization

Based on spectroscopic data and comparison with reported data in the literature (Diastuti et al. 2020), the isolated compound was identified as crotepoxide. This compound appeared as colorless needle crystal; UV(MeOH):λ_{max}=228nm; IR (Atr): 1763 (CO), 1722 (CO), 1280, 1232, 921, 902 cm-1; ^{1}H-NMR (400 MHZ, $CDCl_3$) (Figure 4A): δ 8.01 (2H, dd, J=1.2, 8.4 Hz, H-2' and H-6'), 7.58 (1H, m, H-4'), 7.44 (2H, m, H-3' and H-5'), 5.70 (1H, d, =8.4, H-2), 4.97 (1H, dd, J=2, 9.2 Hz, H-3), 4.55 (1H, d, J=12, H-7), 4.22 (1H, d, J=12, H-7), 3.65 (1H, d, J=2.4, H-6), 3.44 (1H, m, H-5), 3.08 (1H, dd, J=1.2, 4 Hz, H-4), 2.11 (3H, s, CH_3CO at C-11), 2.02 (3H, s, CH_3CO at C-9). ^{13}C-NMR (Figure 4B) showed resonance at δ 170.14 (C=O acetyl at C-10), 169.84 (C=O acetyl at C-8), 165.86 (C=O benzyl at C-7'), 133.65 (C-4'), 129.88 (C-2' and C-6'), 129.19 (C-1'), 128.65 (C-3' and C-5'), 70.45 (C-3), 69.50 (C-2), 62.53 (C- 7), 59.49 (C-1), 53.90 (C-6), 52.70 (C-4), 48.15 (C-5), 20.76 (CH_3 acetyl at C-11), 20.73 (CH_3 acetyl at C-9).

Crotepoxide was initially isolated from the *Croton macrostachyus* and *K. rotunda* (Pancharoen et al. 1996; Diastuti et al. 2020). The IR spectrum of the compound showed absorption bands at 1763 and 1720 $^{cm-1}$, suggesting the presence of C=O stretch values of the benzoate and acetoxy ester groups. In addition, the bands at 1280 and 1232 cm^{-1} indicated the presence of benzoate and acetoxy C-O stretching vibrations. In the fingerprint area, the absorptions of 718 cm^{-1} indicated the presence of a mono-substituted benzene ring, and at 921 and 902 cm^{-1} indicated the presence of epoxy rings. Based on the GC-MS chromatogram isolated compound shows 100% purity and has m/z 362 (Figure 3A). The major fragments obtained from the GC-MS spectra are m/z 105, 77, 51, with a base peak m/z 105 predicted as C_7H_5O (Figure 3B)

The compound contained 18 carbon atoms, 18 hydrogen atoms, and 8 oxygen atoms which were a class of diepoxide cyclohexane compounds (Figure 4C) that are mostly produced in *Kaempferia rotunda* (Pancharoen et al. 1996; Stevenson et al. 2007). The ^{1}HNMR spectrum assignments for the isolated compound are shown in Table 1. The spectrum showed signals for five protons of the aromatic ring at δ 8.01 (2H, m, H-2', H-6'), 7.58 (1H, m, H-4'), and 7.44 (2H, m, H-3', H-5') ppm. Previous-literature reported that the chemical shifts of the protons as doublets

of doublets by Desta and Sewald (2017). However, during our investigation, the ^{1}H-NMR spectra showed protons at δ 8.01 (2H, m, H-2', H-6'), 7.58 (1H, m, H-4'), and 7.44 (2H, m, H-3', H-5') ppm were separated multiplets at different chemical shifts. Two proton signals at δ 2.02 (3H, s, H-9) and 2.11 (3H, s, H-11) ppm were attributed to two methyl of the acetyl groups of the isolated compound. Three proton signals at δ 3.08 (1H, dd, J=1.2, and 4, H-4), 3.44 (1H, m, H-5) and 3.65 (1H, d, J=2.4, H-6) ppm integrating to oxygenated protons, while two proton signals at δ 5.70 (1H, d, J=8.4), and 4.97 (1H, dd, J=2 and 9.2) ppm, were oxygenated protons. Two signals at δ 4.22 (1H, d, J=12) and 4,55 (1H, d, J=12) ppm were two isolated protons whose signals appear as an AB quartet (coupling constant=12 Hz). The ^{13}CNMR spectrum (100 MHz, $CDCl_3$) assignments of the isolated compound are shown in Table 1. The presence of two carbon signals at δ 20.76 (C-11) and 20.73 (C-9) were attributed to two of methyl of acetyl carbons. Six aromatic carbon at δ 128.65 (C-3' and C-5'), 129.19 (C-1'), 129.88 (C-2' and C-6'), and 133.65 (C-4') ppm, while three carbonyl esters at δ 165.87 (c=O benzyl), 169.84 (C=O acetyl at C-8), 170.14 (C=O acetyl at C-10), a methylene carbon at δ 62.53 (C-7), five methine carbons at δ 69.50 (C-2), 70.45 (C-3), 52.70 (C-4), 48.15 (C-5), 53.90 (C-6) and signal of two quarternary carbons at δ 59.49 (C-1) and 129.19 (C-1') ppm.

in vitro antioxidant assay of isolated compound

The antioxidant activity of crotepoxide was evaluated by measuring the abilities of crotepoxide to scavenge free radicals ABTS and DPPH assays and reduce ion (Fe^{3+}) in FRAP assay, respectively; the result is shown in Table 2. The result for ABTS, DPPH, and FRAP assays of isolated compound [38.91 (ABTS); 47.45 (DPPH); and 26.74 (FRAP)] µg/mL indicates lower antioxidant activity compared to that of quercetin [7.20 (ABTS), 28.38 (DPPH), 4.16 (FRAP) µg/mL]. Crotepoxide antioxidant activity on ABTS, DPPH radicals, and reducing ability Ferric (Fe^{3+}) ion show that crotepoxide has IC_{50} value less than 50 µg/mL (38.91; 47.45; and 26.74 µg/mL), so it falls into a very strong antioxidant category, as Marjoni and Zulfisa (2017). These results indicate that crotepoxide was a potential antioxidant for ABTS, DPPH, and FRAP assays, especially for reduction assay. The FRAP assay is based on the capacity of antioxidants to reduce ferri-tripyridyltriazine (Fe^{3+}) to complex ferro-tripyridyltriazine (Fe^{2+}) (Rafi et al. 2020). In addition, crotepoxide has a much higher reducing power than free radicals scavenging. This finding indicates that crotepoxide is suitable as an intercellular antioxidant supplement with a metal chelating rather than a radical scavenging mechanism (Coulibaly et al. 2014).

Antioxidant activity against free radicals was based on the mechanism of Hydrogen Atom Transfer (HAT) and Single Atom Transfer (SET) or a combination of both. The ABTS and DPPH tests were based on a combination of the HAT-SET mechanism, while the FRAP was based on the SET mechanism (Liang and Kitts 2015; Yeap et al. 2017). The crotepoxide structure was enriched by methoxy and carbonyl groups which increased the ability of the compound to donate electrons (Liang and Kitts 2015). The previous report informed that crotepoxide was not active as an anti-radical DPPH (IC_{50}>1000 ppm) (Lotulung et al. 2008), and this is different from the results observed during this study. Therefore, various conditions and treatments may cause this discrepancy, such as DPPH concentration, incubation time, and tested bioactive compound concentrations (Irawan et al. 2022). Meanwhile, the ABTS and FRAP tests were the first studies conducted on crotepoxide isolated from *K. rotunda*.

Crotepoxide was first isolated in 1968 from *Croton macrotachyus* and thereafter after that, for the last ten years, has been isolated from several medicinal plant like *Croton alienus* (Rotich et al. 2022), *Globba pendula* (Ngo et al. 2020), *Kaempferia angustifolia* (Tang et al. 2014; Yeap et al. 2017) and *Kaempferia rotunda* (Diastuti et al. 2020). This compound was first isolated from *Kaempferia rotunda* by Pancharoen (Pancharoen et al. 1996) reported that crotepoxide from *K. rotunda* represented negative values in an antifeedant assay. Based on reported literature, crotepoxide has been investigated for various biological activities like tumor inhibitor, anti-HIV, chemosensitizer tumor cells, anti-leishmanial, anti-bacterial, and antioxidant (Prasad et al. 2010; Diastuti et al. 2020; Rotich et al. 2022).

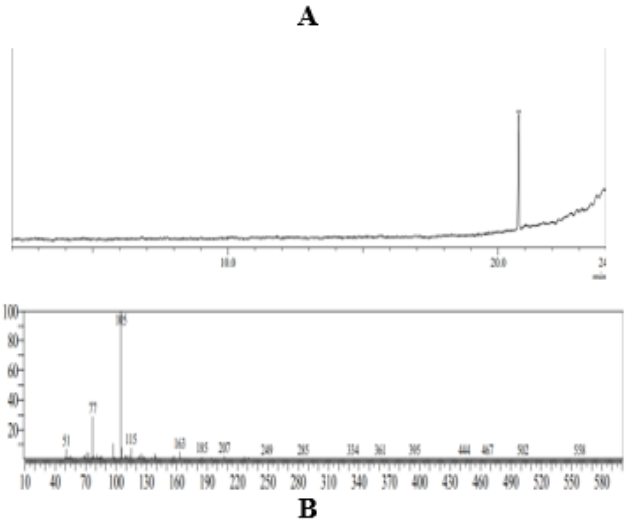

Figure 3. Gas Chromatography-Mass Spectrometry spectra of the isolated compound. A. Gas chromatogram. B. Mass spectrum

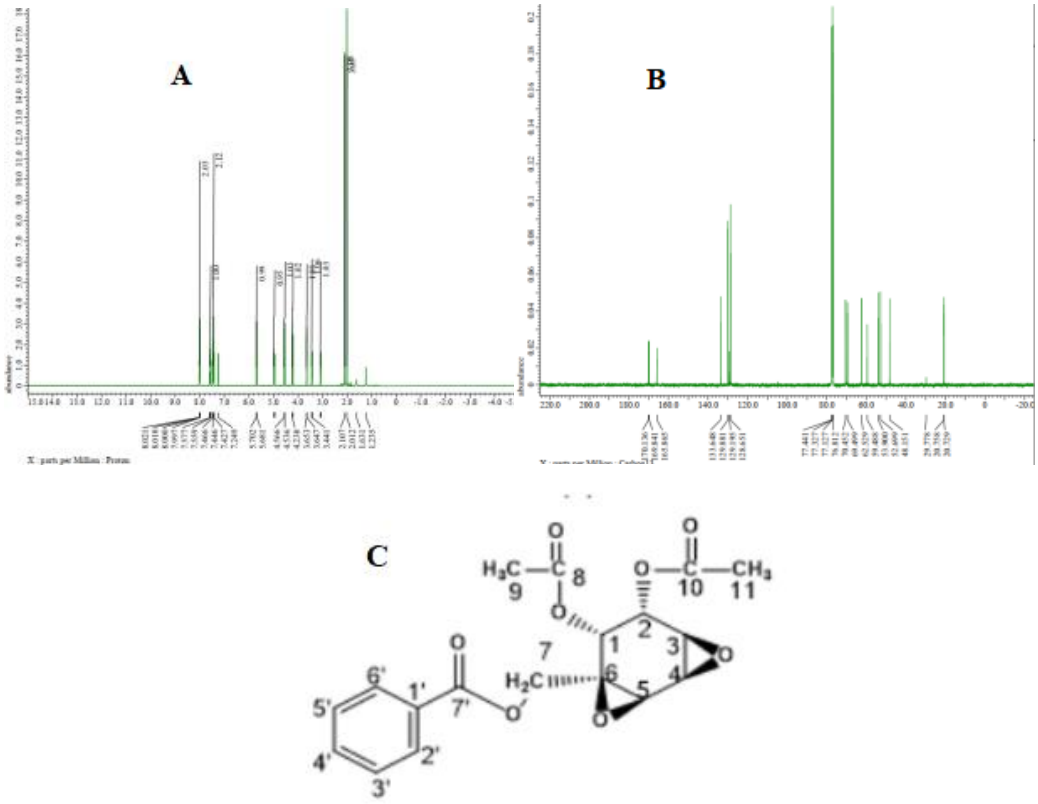

Figure 4. Spectra ($CDCl_3$, 400 MHz). A. ^{1}H-NMR. B. ^{13}C-NM. C. Structure of the isolated compound (Diastuti et al. 2020)

Table 1. ^{1}H-NMR and ^{13}C-NMR chemical shifts of isolated compound

C position	δ_C ppm	δ_H (mult, J=Hz) ppm
1	59.49	-
2	69.50	5.70 (1H, d, 8.4)
3	70.45	4.97 (1H, dd, 2, and 9.2)
4	52.70	3.08 (1H, dd, 1.2, and 4)
5	48.15	3.44 (1H, m)
6	53.90	3.65 (1H, d, 2.4)
7	62.53	4.55 (1H, d, 12) 4.22 (1H. d, 12)
8	169.84	-
9	20.73	2.02 (3H, s) CH3 at acetyl group
10	170.14	-
11	20.76	2.11 (3H, s) CH3 at acetyl group
1’	129.19	-
2’, 6’	129.88	8.01 (2H, m)
3’, 5’	128.65	7.44 (2H, m)
4’	133.65	7.58 (1H, m)
7’	165.87	-

Table 2. Antioxidant activity isolated compound vs. quercetin

Sample	Inhibition concentration (IC50) µg/mL		
	ABTS	DPPH	FRAP
Quercetine	7.20±0.26	28.38±1.03	4.16±0.18
Crotepoxide	38.91±0.5	47.45±0.60	26.74±1.23

In conclusion, the isolation of anti-radical compounds from *K. rotunda* was guided by DPPH anti-radical scavenging bioassay. This study led to the finding of active anti-radical F1 sub-fraction, which yielded an isolated compound. This compound was further identified as crotepoxide based on spectroscopic data and comparison with the reported literature. Crotepoxide bioprospecting was a natural antioxidant compound based on the ABTS, DPPH, and FRAP methods. This compound can maintain ROS imbalance during oxidative stress. So, we suggested that crotepoxide has prospects of being combined with conventional drugs in diabetes mellitus, cataracts, rheumatoid, cancer, atherosclerosis, arthritis, and neurodegenerative diseases.

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