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Research Article
Determination of Quercetin and Flavonol Synthase in *Boesenbergia rotunda* Rhizome

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Abstract

Background and Objective: Flavonols in plants are catalyzed by flavonol synthase (FLS) enzyme. FLS was reported expressed in flowers and fruits, i.e., *Dianthus caryophyllus* L. (Caryophyllaceae), *Petunia hybrida* Hort. (Solanaceae), *Arabidopsis thaliana* L. (Brassicaceae), *Citrus unshiu* Marc. (Rutaceae). However, none reported about FLS in medicinal plants, particularly those which possess anti-inflammatory activity. This study was aimed to extract and identify FLS in the rhizome of *Boesenbergia rotunda* (Zingiberaceae) and to determine quercetin in the ethanol extract of the rhizome. **Materials and Methods:** The protein extraction of the rhizome was carried out by employing Laing and Christeller's (2004) and Wang's (2014) methods. The extracted-proteins were separated by using SDS-PAGE, followed by the measurement of FLS intensity by using Gel Analyzer. The FLS-1 of recombinant *A. thaliana* was employed as the standard. The determination of quercetin in the rhizome was carried out using LC-MS. **Results:** The FLS occurred as a thick band at 38 kDa with intensity 116-158. The LC chromatogram of the extract indicated a small peak at 7.94 min similar to that of quercetin standard. The MS spectra at 7.94 min indicated that quercetin is present in the *B. rotunda* rhizome (m/z = 303.0549). The concentration of quercetin in the extract is 0.022% w/v. **Conclusion:** The FLS, an enzyme which plays an important role in producing quercetin, was detected in *B. rotunda* rhizome planted in Indonesia. As a consequence, quercetin in a small amount, was also quantified in the rhizome of this plant. This report will add a scientific insight of *B. rotunda* for biological sciences.

Key words: Anti-inflammatory activity, *Boesenbergia* sp., flavonols, quercetin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The secondary metabolites of plants, e.g., flavonoids, have been known to represent the plant's pharmacological activity. Flavonols, a small class of flavonoids, have been widely reported to exert anti-inflammatory activity. The FLS catalyzes the conversion of dihydroflavonols to flavonols, i.e., dihydrokaempferol to kaempferol and dihydroquercetin to quercetin, to form a variety of flavonols and glycosidic derivatives[1-3].

Flavonol synthase (FLS) is an enzyme which is contained in flowers, e.g., *Arabidopsis thaliana* L. (Brassicaceae)[1,4,5], *Petunia hybrida* Hort. (Solanaceae)[2], *Dianthus caryophyllus* L. (Caryophyllaceae)[6] and also in fruits, e.g., *Citrus unshiu* Marc. (Rutaceae)[7]. This particular enzyme belongs to the 2-oxoglutarate iron-dependent oxygenase family[8]. The oxidation reaction, which introduced the C2-C3 double bond, was considered to be specific in dihydroflavonol substrates[9].

Extraction of protein from various parts of a plant is considered as a challenging task. There are many obstacles in this process, i.e., the thickness of cellulose walls in the leaves, the secondary metabolites which interfere with the protein, etc[10,11]. However, a few abundant proteins had successfully been identified from the leaves of *Gynura procumbens* by using the proteomic approach. The use of trichloroacetic acid-acetone wash before the protein extraction, could produce distinct protein bands because these solvents rinse out organic-soluble substances, thus precipitate the proteins and other insoluble substances[12,13]. SDS-PAGE, a polyacrylamide gel electrophoresis with a sodium dodecyl sulfate (SDS)-containing buffer, is usually employed to separate the protein. The protein is set apart by the application of an electrical field. The polar part of the buffer is SDS, a negatively-charged amphipathic detergent, while the dodecyl portion, CH_3 $(CH_2)_{11}$, is hydrophobic. When SDS fastens a soluble protein, the hydrophilic portion of the SDS slips into the hydrophobic center of the protein and denatures the protein[14,15].

In this study, SDS-PAGE was employed to extract and to separate protein from the rhizome of *Boesenbergia rotunda* (Zingiberaceae). The quercetin in the ethanol extract of the rhizome was determined by using the standard addition LC-MS method.

MATERIALS AND METHODS

Study area: The study was carried out at the Central Laboratory of Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km 21, West Java, Indonesia, 45363 from April, 2018 to January, 2019.

Plant material and identification: The rhizome of *B. rotunda* was obtained from the Research Institute for Spices and Medicinal Plants (Balittro) Manoko Lembang, West Java, Indonesia (http://balittro.litbang.pertanian.go.id/?p=993&lang=en). The plant was taxonomically identified at the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia (Letter No. 450/HB/10/2017).

Chemicals and other materials: Standard recombinant *A. thaliana* FLS1 (Cusabio Cat. No. # CSB-MP842601DOA), Coomassie blue (Simply Blue™ Safe Stain), Pierce™ silver stain kit (Thermo Scientific Cat. No. #24612), Ladder SeeBlue™ Plus2 pre-stained standard (Invitrogen™ Cat. No. #LC5925), quercetin hydrate (Sigma-AldrichCAS No. 849061-97-8) were purchased from Quartiz Laboratories (a branch of PT Indogen Intertama (http://indogen.id/).

FLS separation and intensity measurement: The rhizome of *B. rotunda* (8 g) was washed with tap water and was ground in liquid nitrogen to a fine powder. About 250 mg of the powder was extracted according to Laing and Christeller[16] and Wang *et al.*[17] methods with a few modifications. The protein was separated using SDS-PAGE for 150 min 80-100 V. The protein bands were stained using Coomassie blue and silver stain compared to that of standard recombinant *A. thaliana* FLS1. The intensity of FLS in the rhizome was calculated using GelAnalyzer (http://www.gelanalyzer.com/).

Extraction of secondary metabolites in the rhizome: About 500 g of the rhizome was macerated in ethanol 95% for 24 h. The macerate was filtered and the residue was re-macerated in ethanol 95% for 2×24 h. The solvent was evaporated at 60-70°C 80 rpm in vacuum until a viscous extract was obtained.

Identification of flavonoids in the ethanol extract of the rhizome using UV spectrophotometry and determination of quercetin using liquid chromatography-mass spectrometry (LC-MS): The presence of quercetin in the ethanol extract of *B. rotunda* (EEBR) was determined according to a method proposed by Bajkacz *et al.*[18] with a few modifications. Quercetin hydrate was used as the standard.

Standard stock solution of quercetin was prepared by dissolving the analyte in ethanol (LC grade) obtaining a concentration of 100 μg mL^{-1}. Working solutions for

calibration standard-addition samples were prepared by diluting the stock solution of quercetin with ethanol and spiked it into the extract solution. This process resulted in the quercetin-extract working solutions of 2.56, 1.28, 0.64 and 0.32 μg mL^{-1}. The 0.32 μg mL^{-1} solution was run on a double-beam spectrophotometry to obtain the spectrum of flavonols in the EEBR.

Eventually, the 2.56, 1.28, 0.64 and 0.32 μg mL^{-1} working solutions were filtered using a 0.2 μm millipore membrane prior to be injected into the Acquity™ Ultra Performance LC BEH shield RP18 1.7 μm, 2.1×100 mm column of Waters Acquity™ Ultra Performance LC in tandem with Waters Xevo™ Qt of MS. Mobile phase used was a mixture of phase A (H_2O-formic acid 0.1%) and phase B (methanol). Flow rate was set to 0.2 mL min^{-1}.

Phytochemical screening and identification of flavonols: Phytochemical screening was carried out as per standard method described by Tiwari *et al.*[19] at the Central Laboratory of Universitas Padjadjaran, Indonesia.

RESULTS

FLS separation and intensity measurement: The standard recombinant *A. thaliana* FLS-1 indicated a thick band at 38 kDa (Fig. 1a). The rhizome of *B. rotunda* indicated several protein bands (Fig. 1b) and a thick band at 38 kDa. The intensity of FLS in *B. rotunda* rhizome is 116-158 (Fig. 2).

Identification of flavonoids in the ethanol extract of the rhizome using UV spectrophotometry: The spectrum of *B. rotunda* extract indicated 2 maxima at 209 and 291 nm (Fig. 3) which confirmed the presence of ring A and ring B of flavonoids.

Determination of quercetin in the ethanol extract of the rhizome using liquid chromatography-mass spectrometry (LC-MS): Liquid chromatography method of EEBR indicated the presence of quercetin (Fig. 4), which was eluted at 7.94 min, similar to quercetin standard. The MS spectrum at 7.94 min revealed the $[M+H]^+$ peak of quercetin standard m/z = 303.0546 (Fig. 5a) and the $[M+H]^+$ peak of quercetin in EEBR m/z = 303.0549 (Fig. 5b). The concentration of quercetin in Indonesian *B. rotunda* is 0.2 mg g^{-1} or 0.022% (linear regression equation).

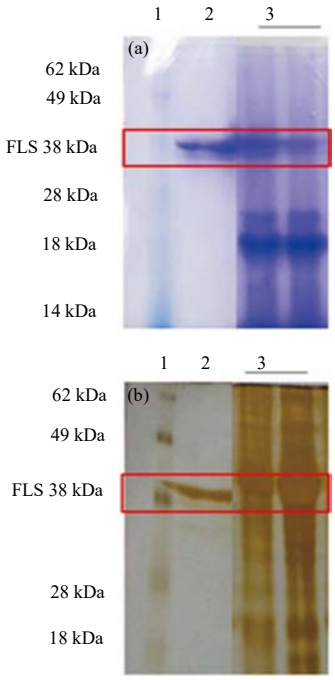

Fig. 1(a-b): SDS-electropherograms of rhizome extract, (a) Coomassie blue and (b) Silver stain were used as staining reagents
1: Ladder, 2: FLS-1 standard, 3: *B. rotunda*, red rectangle indicates the position of FLS band

DISCUSSIONS

The high intensity of FLS in *B. rotunda* is understandable, because this plant has been reported in containing flavonols and other phytochemical constituents[20]. The presence of FLS in *B. rotunda* confirms the conversion of dihydroflavonol to flavonol catalysed by this enzyme. Furthermore, plants belonged to Zingiberaceae family, have been proven in exhibiting anti-inflammatory activity by reducing prostaglandin production. This activity was predicted due to its flavonoids content[21].

Many spectroscopic studies have revealed that flavons (flavonoids with a 2,3-double bond and 4-keto group) and flavonols (flavonoids with a 2,3-double bond, 3-OH and 4-keto groups) possess 2 main absorption bands: band 1 (300-385 nm) belongs to ring B, whereas band 2 (250-295 nm) for ring A. In flavanones (flavonoids with 4-keto groups) and dihydroflavonols (flavonoids with with 3-OH and 4-keto groups), band A appears as a shoulder peak at 300-330 nm and band B, in the 277-295 nm range, is the main peak[22-24].

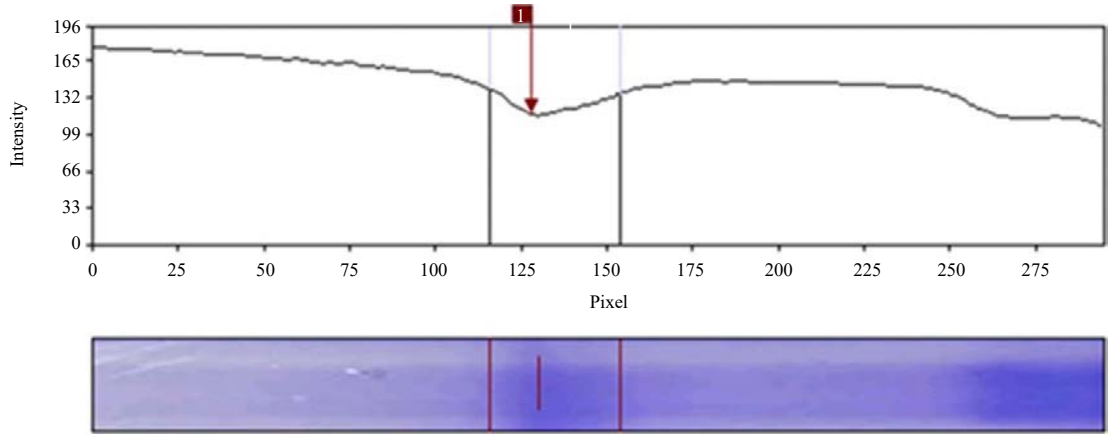

Fig. 2: Intensity of FLS in *B. rotunda* rhizome
Calculated using GelAnalyzer

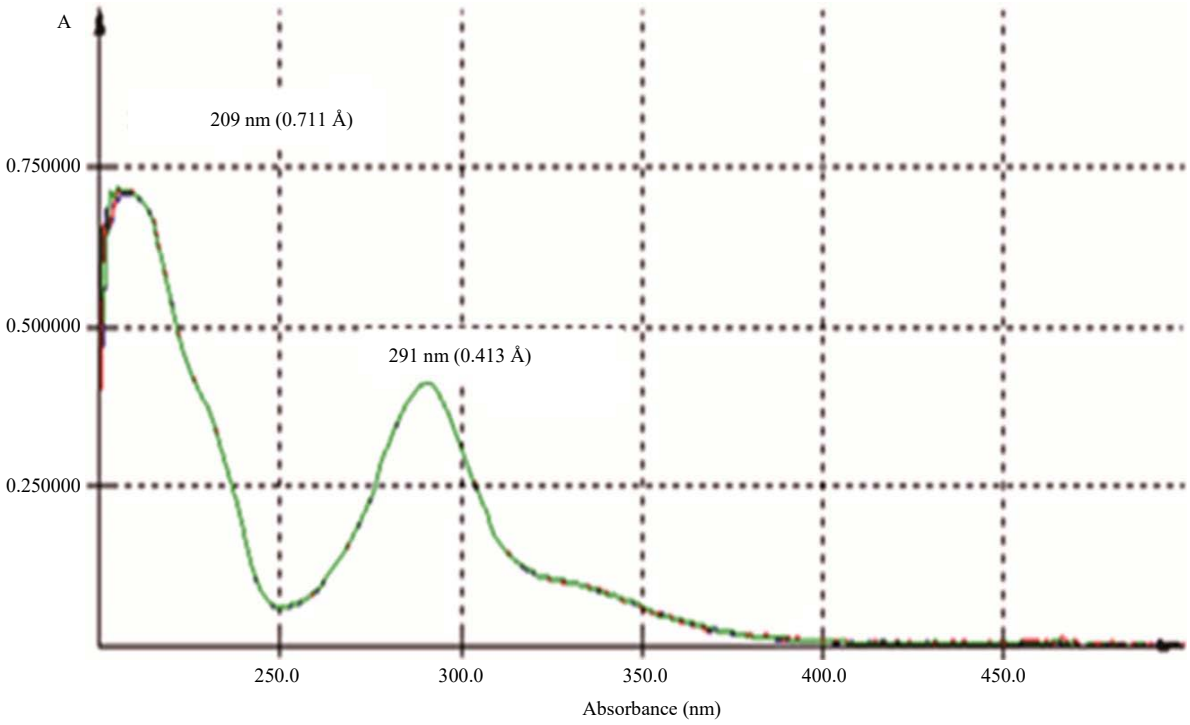

Fig. 3: Ultraviolet absorption spectrum of *B. rotunda* rhizome extract in ethanol (λ_{max} at 209 and 291 nm)

The concentration of quercetin in Indonesian *B. rotunda* is 0.2 mg g^{-1} or 0.022% is less compared to Malaysian *B. rotunda* (0.58 mg g^{-1})[20]. Quercetin was also reported contained in *Zingiber zerumbet* (3.94 mg g^{-1}), *Z. officinale* (3.66 mg g^{-1}), *Alpinia galanga* (29.535 mg g^{-1}), *A. officinarum* (33.66 mg g^{-1})[19,24,25]. Flavonoids, e.g., anthocyanins, flavones, isoflavones, flavonols, etc., have been extensively studied[26-29]. The pathway of flavonoid biosynthesis has been well understood and the key biosynthetic enzymes have been identified[29-31].

Quercetin, although presents in a low quantity in the rhizome of *B. rotunda*, might contribute to this plant's activity. Further exploration on this plant's pharmacological activity, is still needed, to develop this plant as the future plant-based therapy.

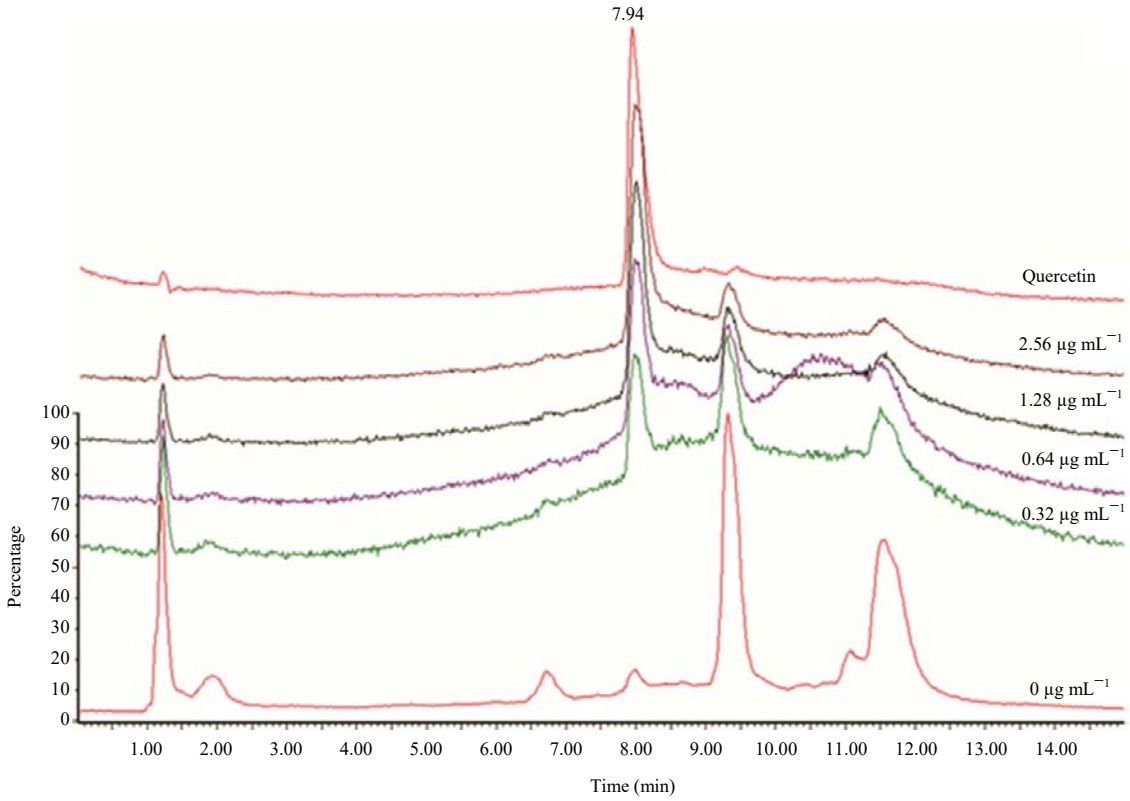

Fig. 4: LC chromatograms of quercetin-spiked EEBR

Quercetin is eluted at 7.94 min

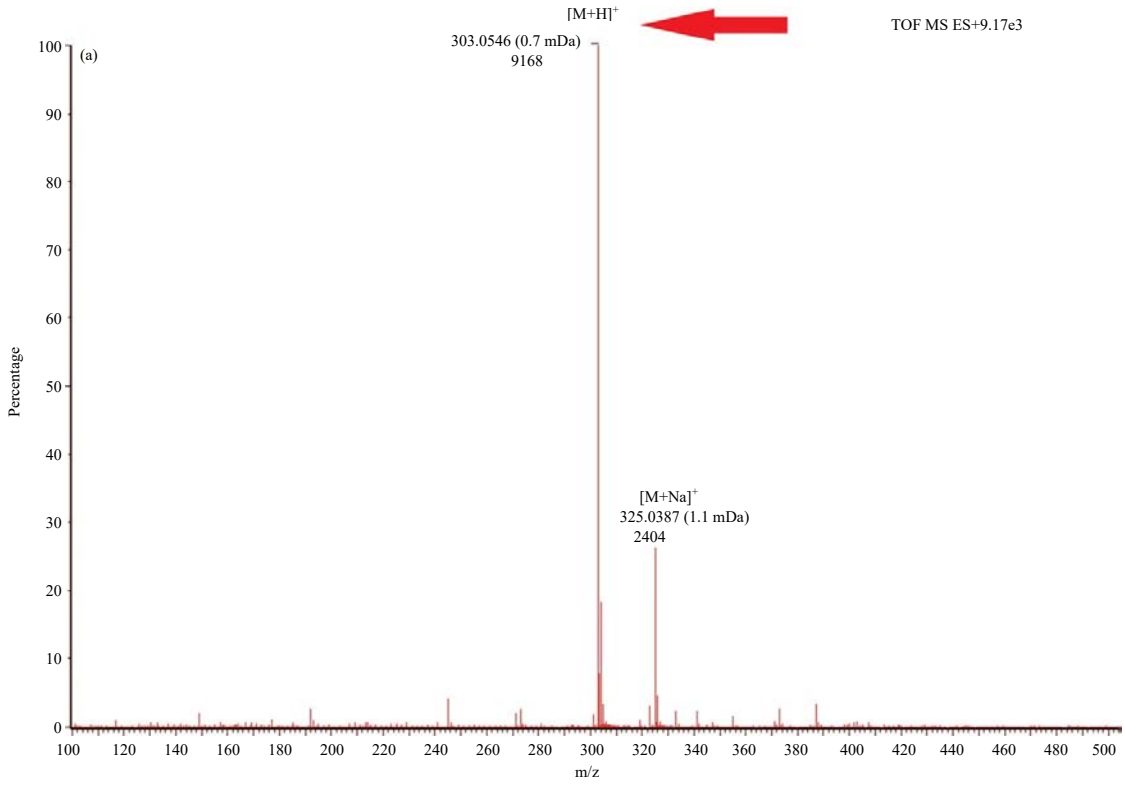

Fig. 5(a-b): Continue

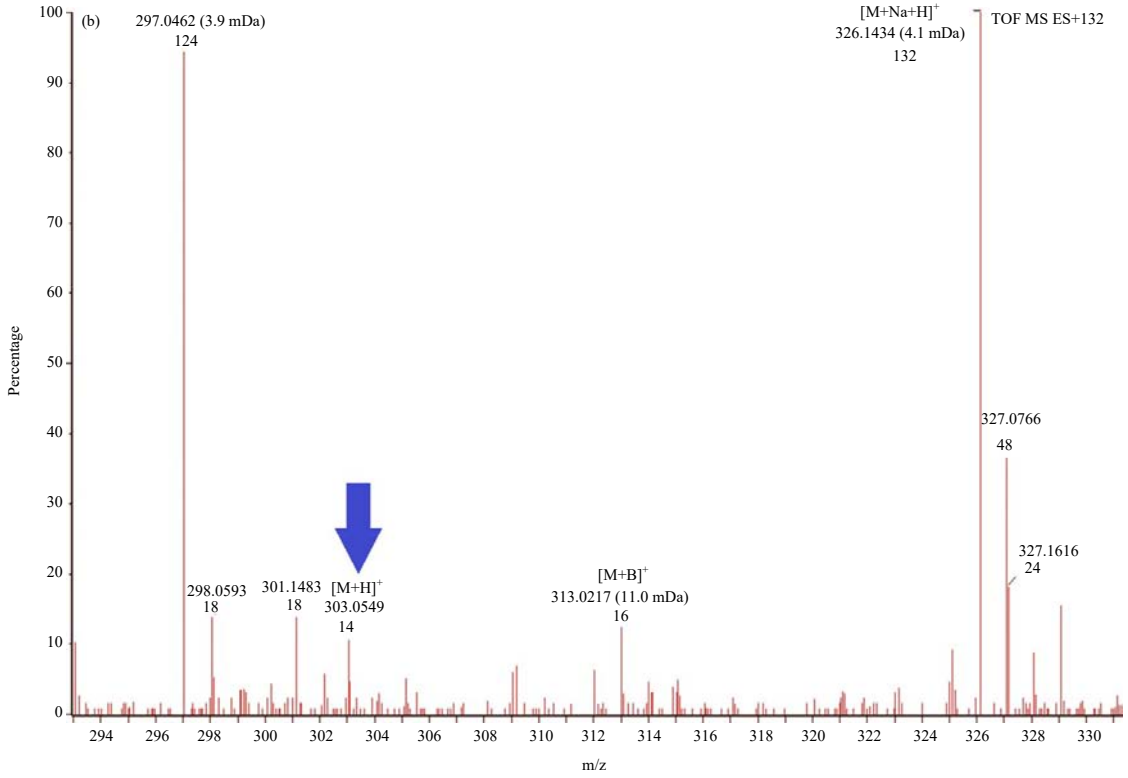

Fig. 5(a-b): Mass spectrum of (a) Quercetin standard $[M+H]^+$ m/z = 303.0546 (indicated by red arrow) and (b) Quercetin in EEBR $[M+H]^+$ m/z = 303.0549 (indicated by blue arrow)

CONCLUSION

Flavonol synthase, an enzyme which plays an important role in producing quercetin, was detected in *B. rotunda* rhizome planted in Indonesia. As a consequence, quercetin in a small amount, was also quantified in the rhizome of this plant. This report will add a scientific insight of *B. rotunda* for biological sciences.

SIGNIFICANCE STATEMENT

This study discovers the presence of flavonol synthase enzyme and quercetin in *Boesenbergia rotunda* rhizome planted in Indonesia, that can be beneficial for the development of traditional medicinal plants and drug discovery. This study will help the researcher to uncover the critical area of *B. rotunda*'s bioactivity that many researchers were not able to explore. Thus, a new theory on the plant's pharmacological activity, may be arrived at.

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