Metabolite Detection and Antibacterial Activity of Fungal Endophytic Extracts Isolated from Brotowali (Tinospora crispa) Plants using TLC-Bioautography Assay

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Abstract. Endophytic fungi may be a potent source of bioactive compounds and have a vast repertoire of diverse metabolites. One source of endophytic fungi host plants is a medicinal plant such as Brotowali or Tinospora crispa. Research corresponding to the antibacterial activity of endophytic fungi extracts isolated from T.crispa taken from several regions in West Java has never been reported yet. While antibacterial methods to evaluate their activity as well as to identify the chemical compounds is a TLC-bioautography assay. This research is aimed to investigate the components of endophytic fungal extracts and their antibacterial activity using the TLC-bioautography assay method. In this study, eighty isolates of endophytic fungi have been successfully isolated from plant tissues of T.crispa from several regions in West Java. Antibacterial activity by using TLC (dot-blot) plates revealed that the fungal extracts could inhibit S. aureus (77 extracts) and E. coli (35 extracts) with inhibition zones ranging from 8-30 mm. The endophytic fungi extracts that showed potent antibacterial activity against Gram-positive bacteria (S.aureus) were sixteen extracts, while nine extracts have good inhibition activity against Gram-negative bacteria (E.coli). Among the fungal extracts that have excellent ability against both Gram-positive and negative was namely TcBt1Bd-10 extract. Based on metabolites analysis using the TLC method, the possibility of the chemical compounds that played a role in antibacterial activity of the extracts, including phenolics, flavonoids, alkaloids, and terpenoids.

1. Introduction
Endophytic fungi may be an excellent source of bioactive compounds and have a vast repertoire of diverse metabolites [1]. Endophytic fungi are known as symbiotically associated with the plant tissues, and they induce without symptoms to their hosts [2]. Due to they are capable of synthesizing bioactive metabolites that can be used for their host plant defence against fungal and bacterial pathogenic, so endophytic fungi also have known as “the chemical synthesizers inside plants” [3]. The fungal bioactive compounds produced have important pharmacological activities such as antioxidant, anticancer, immunomodulatory, antivirus, antituberculosis, antiparasite, and insecticides [4]. One source of endophytic fungi is a medicinal plant such as Brotowali or Tinospora crispa. The extracts of T.crispa plants have known as the potential multiple pharmacological and therapeutic activities due to the presence of various chemical compounds in the herb [5]. Chromatography methods can separate various
bioactive compounds. Thin-layer chromatography analysis performed with biological activity detection is termed bioautography. TLC-bioautography assay is a fast and inexpensive method [6]. Bioautography assay belongs to screening methods commonly used for antimicrobial activity detection [7] and enables the search for biologically active substances in complex mixtures [8] such as endophytic fungi, plant, and actinomycetes extracts [9, 10, 11]. The principle of this method is the separation and detection of components of extracts which are carried out directly on a TLC plate. The extracts were transferred on a TLC plate and developed with a suitable solvent, then observed using both viewed under UV-light and sprayed staining-reagents to identify the secondary metabolites. The determination of antibacterial activity was also carried out using TLC dot-blot and TLC-bioautography assay, then dipped in bacterial culture, and bacteria grew directly on the layers during the incubation time. Inhibition zones are formed in the places where the antibacterial component is located. This study aims to investigate the components of endophytic fungal extracts and their antibacterial activity using the TLC-bioautography assay method.

2. Materials and Methods

2.1. Material
Samples of fresh and healthy parts of *T. crispa* plants (stem, leaves, and petiole) were collected from Bogor, Bekasi, and Bandung, West Java, Indonesia. Identification of the plant specimen was carried out at Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences (LIPI).

2.2. Staining-reagents
Dragendorff (Merck) reagent, 0.25% vanillin (Sigma-Aldrich) solution in 10% sulphuric acid-ethanol, 1% Ce(SO₄)₂ (Merck) solution in 10% sulphuric acid-ethanol, folin-ciocalteu (Merck) reagent. After sprayed vanillin-sulphuric acid or cerium (IV) sulfate, the TLC plates (Silica gel 60 F₂₅₄, Merck) were heated at 110 °C for optimal color visualization. All reagents and solvents used in this research were analytical grades.

2.3. Bacterial Isolates for Antibacterial Testing
Bacterial isolates, namely *S. aureus* InaCC-B4 and *E. coli* InaCC-B5, were used for the antibacterial test. They are microbial collections of Indonesian Culture Collection (InaCC), Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI).

2.4. Isolation of Endophytic Fungi
Fungal endophytic isolation was carried out by surface sterilization, according to Praptiwi et al. [9] with minor modification. Healthy and fresh parts of plants (stems, leaves, and petiole) collected from the field were stored at a temperature of 4°C. These samples were cleaned under tap water and dipped in 70% ethanol for 1 minute, then dipped in 5.3% sodium hypochlorite for 2.5-3.5 minutes and finally dipped in 70% ethanol for 30 seconds. Samples were dried under aseptic conditions. The sterilized samples were cut aseptically into around size of 1×1 cm², and then, placed on top of the Corn Meal Malt Agar (CMMA) growth medium containing 17 g/L corn meal agar (Fluka, Sigma-Aldrich), 20 g/L malt extract (Bacto™), and 2 g/L yeast extract (Bacto™) were supplemented with 0.05 g/L chloramphenicol (Sigma), and then incubated at room temperature for 3-5 days. The emerging colonies were subcultured several times on Potato dextrose agar (PDA, Difco™) until a single isolate was obtained. Pure isolates were preserved in 10% glycerol and stored at -80°C at the Indonesian Culture Collection (InaCC), Indonesian Institute of Sciences (LIPI).

2.5. Cultivation and Extraction of Endophytic Fungi
Each fungal isolate was cultured on potato dextrose broth (PDB, Difco™) (@200 ml in culture flask of 500 ml) and incubated in a dark room at a temperature of 25-26°C for 21 days. After the incubation period is completed, both growth media and fungal biomass were extracted with ethyl acetate (EtOAc).
The extract solvent was removed under reduced pressure, and the dry extracts were stored in the glass vials at -30°C until used.

2.6. Chemical Compounds Analysis: Thin Layer Chromatography (TLC) Detection
Chemical compounds analysis of fungal extracts were performed on silica gel Thin-layer chromatography (TLC) plates (silica gel 60 F<sub>254</sub>, Merck). The dried extract was prepared in 10 mg/ml, and ten microliters of extract were transferred on the TLC plate and developed in CH<sub>2</sub>Cl<sub>2</sub>: MeOH (10:1). Separated chemical compounds were visualized under UV light of 254 and 366 nm (Camag) and followed by spraying with staining-reagent dragendorff, or vanillin-sulfuric acid, or cerium (IV) sulfate, or folin-ciocalteu.

2.7. Screening of Antibacterial Activity: TLC Dot-Blot Assay
Antibacterial Activity screening by using the TLC Dot-Blot assay was carried out to assess of antibacterial potency of fungal extracts. Ten microliters of fungal extracts were loaded on the TLC plates (Silica gel 60 F<sub>254</sub>, Merck) and then dried at room temperature. The TLC Plate then immersed into the suspension of bacterial culture, then incubated the plate in a humid chamber for 18-24 hours at a temperature of 37 °C. After the incubation period was finished, the plates were sprayed with iodonitrotetrazolium p-violet (INT, Sigma). The inhibition of bacteria growth was viewed by the formation of a clear zone around the active extract. Then the inhibition zone of the active extract was measured as inhibition zone diameter. Then, the active extracts were further evaluated the antibacterial activity by using a TLC-direct bioautography assay.

2.8. TLC-Direct Bioautography Assay
Ten microliters of the selected extracts were loaded on the TLC plates and then dried at room temperature. Then the active extract on the TLC plate developed with mobile phase CH<sub>2</sub>Cl<sub>2</sub>: MeOH (10:1) and dried at room temperature. The TLC Plate then immersed into the suspension of bacterial culture, then incubated the plate in a humid chamber for 18-24 hours at a temperature of 37 °C. After the incubation period was finished, the plates were sprayed with iodonitrotetrazolium p-violet (INT, Sigma). The inhibition of bacteria growth was viewed by the formation of a clear zone around the active band of the chemical compounds contained in the extracts. Then inhibition band of the active compounds was measured as Retention factor (Rf).

3. Results and Discussion
Eighty isolates of endophytic fungi were successfully obtained from healthy and fresh tissues of T.crispa plants. Fungal endophytic isolates were associated with T.crispa plants, i.e., 30, 26, and 24 isolates from stem leaves and petioles, respectively (Table 1). Another study, Sun et al. [12] reported that host plant species and tissue types significantly affected the endophytic fungi community, where the overall colonization rate of endophytic fungi was significantly higher in branches than in leaves. This study showed that the host plant, T.crispa, was taken from Bogor and obtained the most endophytic fungal isolates, probably due to differences in geographic location and abiotic factors. Several endophytic fungi isolated from T.crispa after cultivated in PDB media for 21 days (Figure 1) with distinct exudate colors in broth media showed the different and diverse fungal species. According to a previous study, the rate of endophytic fungal colonization, diversity, and community composition was influenced by host plant species, genotypic, tissue types, geographic location, and abiotic factors [13, 12, 14, 15, 16]. The composition of endophytic fungal communities influenced by their host plant species and tissue types, and some endophytic fungi showed significant host and tissue preferences [17].
**Table 1. Endophytic Fungi associated with *T.crispa***

| No. Sample | Isolate Name | Origin plant from | Plant part | Weight of EtOAc extracts* (mg) | No. Sample | Isolate Name | Origin plant from | Plant part | Weight of EtOAc extracts* (mg) |
|------------|--------------|--------------------|------------|-------------------------------|------------|--------------|--------------------|------------|-------------------------------|
| 1          | TcBt1Bd-02   | Bandung            | Stem       | 69.5                          | 41         | TcTd2Be-04   | Bekasi            | Petiole    | 27.2                          |
| 2          | TcBt1Bd-05   | Bandung            | Stem       | 99.9                          | 42         | TcBt1Bo-01   | Bogor             | Stem       | 66.5                          |
| 3          | TcBt1Bd-07   | Bandung            | Stem       | 131.0                         | 43         | TcBt1Bo-02   | Bogor             | Stem       | 154.3                         |
| 4          | TcBt1Bd-08   | Bandung            | Stem       | 182.1                         | 44         | TcBt1Bo-03   | Bogor             | Stem       | 112.4                         |
| 5          | TcBt1Bd-09   | Bandung            | Stem       | 108.4                         | 45         | TcBt1Bo-04   | Bogor             | Stem       | 82.2                          |
| 6          | TcBt1Bd-10   | Bandung            | Stem       | 62.2                          | 46         | TcBt1Bo-05   | Bogor             | Stem       | 122.7                         |
| 7          | TcBd2Bd-01   | Bandung            | Stem       | 226.2                         | 47         | TcBt1Bo-06   | Bogor             | Stem       | 233.1                         |
| 8          | TcBd2Bd-03   | Bandung            | Stem       | 72.0                          | 48         | TcBt1Bo-07   | Bogor             | Stem       | 241.0                         |
| 9          | TcBd2Bd-04   | Bandung            | Stem       | 85.9                          | 49         | TcBt1Bo-08   | Bogor             | Stem       | 73.9                          |
| 10         | TcBd2Bd-06   | Bandung            | Stem       | 33.0                          | 50         | TcBt1Bo-09   | Bogor             | Stem       | 40.0                          |
| 11         | TcDn1Bd-01   | Bandung            | Leaf       | 102.5                         | 51         | TcBt1Bo-10   | Bogor             | Stem       | 134.0                         |
| 12         | TcDn1Bd-02   | Bandung            | Leaf       | 52.2                          | 52         | TcBt2Bo-01   | Bogor             | Stem       | 91.8                          |
| 13         | TcDn1Bd-03   | Bandung            | Leaf       | 37.9                          | 53         | TcBt2Bo-02   | Bogor             | Stem       | 23.7                          |
| 14         | TcDn2Bd-01   | Bandung            | Leaf       | 18.8                          | 54         | TcBt2Bo-03   | Bogor             | Stem       | 134.8                         |
| 15         | TcDn2Bd-02   | Bandung            | Leaf       | 38.0                          | 55         | TcBt2Bo-04   | Bogor             | Stem       | 60.9                          |
| 16         | TcDn2Bd-03   | Bandung            | Leaf       | 70.8                          | 56         | TcDn1Bo-01   | Bogor             | Leaf       | 31.0                          |
| 17         | TcDn2Bd-04   | Bandung            | Leaf       | 69.6                          | 57         | TcDn1Bo-02   | Bogor             | Leaf       | 32.3                          |
| 18         | TcTd1Bd-01   | Bandung            | Petiole    | 90.5                          | 58         | TcDn1Bo-03   | Bogor             | Leaf       | 27.1                          |
| 19         | TcTd1Bd-02   | Bandung            | Petiole    | 32.8                          | 59         | TcDn1Bo-04   | Bogor             | Leaf       | 89.5                          |
| 20         | TcTd1Bd-03   | Bandung            | Petiole    | 104.1                         | 60         | TcDn1Bo-05   | Bogor             | Leaf       | 14.7                          |
| 21         | TcTd2Bd-01   | Bandung            | Petiole    | 92.0                          | 61         | TcDn2Bo-01   | Bogor             | Leaf       | 31.0                          |
| 22         | TcTd2Bd-02   | Bandung            | Petiole    | 178.3                         | 62         | TcDn2Bo-02A  | Bogor             | Leaf       | 61.0                          |
| 23         | TcTd2Bd-03   | Bandung            | Petiole    | 118.0                         | 63         | TcDn2Bo-03   | Bogor             | Leaf       | 19.2                          |
| 24         | TcTd2Bd-04   | Bandung            | Petiole    | 98.0                          | 64         | TcDn2Bo-04   | Bogor             | Leaf       | 100.4                         |
| 25         | TcTd2Bd-05   | Bandung            | Petiole    | 13.5                          | 65         | TcDn2Bo-05   | Bogor             | Leaf       | 130.7                         |
| 26         | TcTd2Bd-06   | Bandung            | Petiole    | 139.2                         | 66         | TcDn2Bo-06   | Bogor             | Leaf       | 31.1                          |
| 27         | TcTd1Be-01   | Bekasi             | Stem       | 126.8                         | 67         | TcDn2Bo-07   | Bogor             | Leaf       | 50.0                          |
| 28         | TcTd1Be-03   | Bekasi             | Stem       | 79.0                          | 68         | TcDn2Bo-08A  | Bogor             | Leaf       | 151.9                         |
| 29         | TcBt2Be-01   | Bekasi             | Stem       | 17.1                          | 69         | TcDn2Bo-09   | Bogor             | Leaf       | 76.2                          |
| 30         | TcBt2Be-05   | Bekasi             | Stem       | 29.3                          | 70         | TcTd1Bo-01   | Bogor             | Petiole    | 38.2                          |
| 31         | TcBt2Be-06   | Bekasi             | Stem       | 73.1                          | 71         | TcTd1Bo-02   | Bogor             | Petiole    | 24.4                          |
| 32         | TcBt2Be-07   | Bekasi             | Stem       | 79.2                          | 72         | TcTd1Bo-03   | Bogor             | Petiole    | 18.2                          |
| 33         | TcDn1Be-01   | Bekasi             | Leaf       | 33.9                          | 73         | TcTd1Bo-04   | Bogor             | Petiole    | 35.8                          |
| 34         | TcDn2Be-01   | Bekasi             | Leaf       | 57.1                          | 74         | TcTd2Bo-01A  | Bogor             | Petiole    | 24.8                          |
| 35         | TcDn2Be-02   | Bekasi             | Leaf       | 34.1                          | 75         | TcTd2Bo-02   | Bogor             | Petiole    | 28.8                          |
| 36         | TcDn2Be-03   | Bekasi             | Leaf       | 52.1                          | 76         | TcTd2Bo-03   | Bogor             | Petiole    | 58.5                          |
| 37         | TcDn2Be-04   | Bekasi             | Leaf       | 15.1                          | 77         | TcTd2Bo-04   | Bogor             | Petiole    | 67.0                          |
| 38         | TcTd1Be-02   | Bekasi             | Petiole    | 383.8                         | 78         | TcTd2Bo-05   | Bogor             | Petiole    | 132.3                         |
| 39         | TcTd2Be-02   | Bekasi             | Petiole    | 33.0                          | 79         | TcTd2Bo-06   | Bogor             | Petiole    | 104.9                         |
| 40         | TcTd2Be-03   | Bekasi             | Petiole    | 224.0                         | 80         | TcTd2Bo-07   | Bogor             | Petiole    | 68.9                          |

**Remark:** (*) Extracts produced by 200 ml endophytic fungi cultured in PDB media, static condition, for 21 days.
Figure 1. Some Endophytic Fungi isolated from *T.crispa* plant (cultured in PDB media, 21 days). A: TCBt1Bo-1, B: TCBt1Bo-10, C: TCBt1Be-1, D: TCTd2Bd-4, E: TCBt1Bo-6, F: TCTd1Bo-4, G: TCBt2Bd-6, H: TCBt2Bd-1.

In this study, several flavonoids without chemical treatment appeared red color under visible light, and some terpenoids and coumarins showed blue fluorescent and dark zones in the chromatogram that viewed under UV of 254 nm (Figure 2A and 2B). While, some flavonoids viewed under 366 nm shown yellow or dark yellow, or brown fluorescence (Figure 2C). The spray-reagents used to allow the composition of the fungal extracts. General staining-reagent and very well for the detection of alkaloid compounds is cerium (IV) sulfate that shown brown color (Figure 2D). Another general spray-reagent and suitable for the detection of hydroxyl or carbonyl compounds is vanillin-sulfuric acid that appeared diverse colors, i.e., red (phenolics), and green (terpenoids) (Figure 2E). Staining-reagent by using dragendorff, alkaloids appear as a stable orange color (visible) (Figure 2F). Folin-ciocalteu reagent used for phenolic compound detection that shown dark-blue color (Figure 2H). The standard of metabolites used berberine (alkaloid), phloroglucinol (phenolic), and quercetin (flavonoid) appeared brown (without treatment, and viewed under UV 254 nm), red (after sprayed vanillin), and dark zone (after sprayed folin-ciocalteu) (unpublished data) (Table 2). Phenolics compound (gallic acid) in other studies showed a blue or dark zone [24], but the phenolic standard (phloroglucinol) did not appear dark or blue (unpublished data). While dark or blue color appeared for the detection of flavonoid (quercetin standard) after sprayed folin-ciocalteu. From this study, folin-ciocalteu possible allow as staining-reagent for flavonoid compound detection by the TLC method.

In the previous study reported that endophytic fungi extract isolated from *T.crispa* could have produced diverse metabolites such as flavonoids, phenolics, terpenoids, and coumarins. While an alkaloid (cytochalasin D) and phenolic (phloroglucinol) produced by an endophytic fungus that isolated from *Albertisia papuana* leaves [24, 25], Praptiwi et al. [26] and Agusta et al. [27] reported that fungal endophytic associated with *Uncaria gambir* stems could produce anthraquinone compounds ((+)-1,1'-bislunatin and (+)-2,2'-epicytoskyrin A). Two alkaloids are produced by endophytic fungus isolated from *Annona squamosa* plant [28]. Fungal metabolites such as steroids were produced by endophytic fungus isolated from *Cynodon dactylon* leaves [29], and some sesquiterpene quinones were isolated from a fungal strain endophytic fungus associated with *Platycladus orientalis* plant [30]. Bioactive compounds such as some coumarins were isolated from an endophytic fungus that associated with the leaves of the Chinese mangrove *Rhizophora mucronate* [31].
Table 2. Metabolite detection by using UV light and spray-reagent.

| UV light /Staining Reagent | Detection for Compounds with extended conjugation (aromatic compounds) | Colors (metabolites) | Literatures |
|---------------------------|---------------------------------------------------------------------|---------------------|-------------|
| UV light (254 nm)         | Blue-white (coumarins) Dark zones (terpenoids with conjugated double bonds) | Dark zones (flavonoid: quercetin standard, Sigma) | [18, 19] |
| UV light (365 or 366 nm)  | Yellow, or brown, or blue, or blue-green fluorescence (coumarins) | Yellow, or orange, or red fluorescence (all anthracene derivatives) | [18, 19, 20] |
| Cerium (IV) sulfate       | Yellow (alkaloid : berberine standard, Merck) Brown (alkaloid: cytochalasin D) | Unpublished data |
| Vanillin-H$_2$SO$_4$      | Orange (flavonoid: quercetin standard) Blue-violet, or red (saponins) | Red or yellow or brown or blue-green (flavonoid glycoside) | [19, 20, 21] |
| Dragendorff               | Red (phenolic: phloroglucinol standard) | Orange or reddish-brown (alkaloids) | [22, 23] |
| Folin-ciocalteu           | Blue or dark zones (phenolics) Dark zones (flavonoid: quercetin standard) | Unpublished data |

Antibacterial activity by using TLC Dot-Blot assay revealed that six endophytic fungi extracts associated with T. crispa showed potent antibacterial activity (the inhibition zone diameter range of 17-30 mm or > 16 mm) against both S. aureus (Gram-positive) and E. coli (Gram-negative), while some extracts had strong activity only against Gram-positive or Gram-negative bacteria (Table 3). Then all of the active extracts were further evaluated for antibacterial activity test by using the TLC-direct bioautography assay. This study revealed that the alkaloids (Rf: 0.800), phenolics (Rf: 0.125-0.500), and flavonoids (0.500-0.625) produced by endophytic fungi No. 27 and 28 showed antibacterial against only Gram-positive bacteria (Figure 2 and 3). A previous study reported that bioactive metabolites such as alkaloid, pseurotin A, produced by fungal endophytic isolated from Bauhinia guianensis plant had potent activity against B.subtilis, E.coli, P.aeruginosa, and S.aureus [33]. While, phenolic compound, 4-(2,4,7-trioxo-bicyclo [4.1.0] heptane-3-yl) phenol, was produced by endophytic fungus isolated from Mangifera indica plant has strong not only as an antibacterial against Gram-positive and negative bacteria but also antifungal activity [34].
**Figure 2.** Some chromatograms of fungal endophytic extracts (no.1-39). A: under visible light, B: under UV light (254 nm), C: under UV light (366 nm), D: after sprayed staining-reagent (cerium sulfate), E: after sprayed staining-reagent (vanillin-sulfuric acid), F: after sprayed staining-reagent (dragendroff), G: after sprayed staining-reagent (folin-ciocalteu). Mobile phase: CH$_2$Cl$_2$-MeOH (10 : 1). Stationary phase: Silica gel 60 GF$_{254}$ (Merck). Metabolite analysis based on band color according to the previous studies [18, 19, 20, 22, 23, 24].
Table 3. Screening Antibacterial Activity of Fungal Endophytic Extracts.

| No Sample | Isolate Name | Region origin | Plant Parts | IZD against \textit{S.aureus} (mm) | Category of Antibacterial against \textit{S.aureus} | IZD against \textit{E.coli} (mm) | Category of Antibacterial against \textit{E.coli} |
|-----------|--------------|---------------|-------------|-------------------------------------|-----------------------------------------------|---------------------------------|-----------------------------------------------|
| 6         | TcBt1Bd-10   | Bandung       | Stem        | 30                                  | Strong                                        | 30                              | Strong                                        |
| 1         | TcBt1Bd-02   | Bandung       | Stem        | 22                                  | Strong                                        | 23                              | Strong                                        |
| 31        | TcBt2Be-06   | Bekasi        | Stem        | 22                                  | Strong                                        | 20                              | Strong                                        |
| 37        | TcDn2Be-04   | Bekasi        | Leaf        | 21                                  | Strong                                        | 18                              | Strong                                        |
| 8         | TcBt2Bd-03   | Bandung       | Stem        | 19                                  | Strong                                        | 17                              | Strong                                        |
| 68        | TcDn2Bo-08A  | Bogor         | Leaf        | 19                                  | Strong                                        | 17                              | Strong                                        |
| 67        | TcDn2Bo-07   | Bogor         | Leaf        | 22                                  | Strong                                        | 10                              | Weak                                          |
| 80        | TcTd2Bo-07   | Bogor         | Petiole     | 22                                  | Strong                                        | NA                              | Not active                                    |
| 32        | TcBt2Be-07   | Bekasi        | Stem        | 20                                  | Strong                                        | 14                              | Moderate                                      |
| 47        | TcBt1Bo-06   | Bogor         | Stem        | 20                                  | Strong                                        | 15                              | Moderate                                      |
| 28        | TcBt1Bo-03   | Bekasi        | Stem        | 19                                  | Strong                                        | NA                              | Not active                                    |
| 51        | TcBt1Bo-10   | Bogor         | Stem        | 19                                  | Strong                                        | 15                              | Moderate                                      |
| 53        | TcBt2Bo-02   | Bogor         | Stem        | 19                                  | Strong                                        | NA                              | Not active                                    |
| 57        | TcDn1Bo-02   | Bogor         | Leaf        | 19                                  | Strong                                        | NA                              | Not active                                    |
| 10        | TcBt2Bd-06   | Bandung       | Stem        | 17                                  | Strong                                        | NA                              | Not active                                    |
| 38        | TcTd1Be-02   | Bekasi        | Petiole     | 17                                  | Strong                                        | NA                              | Not active                                    |
| 16        | TcDn2Bd-03   | Bandung       | Leaf        | 15                                  | Moderate                                      | 20                              | Strong                                        |
| 22        | TcTd2Bd-02   | Bandung       | Petiole     | 14                                  | Moderate                                      | 19                              | Strong                                        |
| 45        | TcBt1Bo-04   | Bogor         | Stem        | 14                                  | Moderate                                      | 20                              | Strong                                        |

Remark: IZD: Inhibition Zone Diameter. Selected data (strong activity against \textit{S. aureus} and/or \textit{E.coli}). Antibacterial activity category based on the diameter of inhibition zone: 7-10 mm (weak); 11-15 mm (moderate); >16 mm (strong) [32].

Figure 3. Bioautogram of selected extracts (active extracts against \textit{S.aureus} and \textit{E.coli}). Mobile phase: CH$_2$Cl$_2$-MeOH (10 : 1). Stationary phase: Silica gel 60 GF$_{254}$ (Merck). I: active extracts against \textit{S.aureus}, II: active extracts against \textit{E.coli}. 
In addition, some flavonoids such as 3-methoxy flavone, nobiletin, formononetin, scopoletin, and daidzein contained in endophytic fungi that have antioxidant and antibacterial activities [35]. One of the endophytic extracts that good antibacterial activity against both Gram-positive and negative bacteria is isolate extract No. 22 showed the terpenoid compounds with Rf of 0.400 and 0.625 as bioactive compounds. In another study, some terpenoid compounds such as conidiogenon B and conidiogenol had excellent antibacterial Gram-positive and negative bacteria [36]. Fungal extract No. 6 was the best extract as antibacterial with Rf around of 0.125-0.750. Identification of bioactive metabolites produced by fungal isolate No. 6 is not very clear, because the band color of compounds is not obviously visible (Figure 2). The limitation of component analysis by using TLC methods for the compound with unclear color bands, but this method provides a fundamental separation of compounds as well as bioassay-guided isolation for compounds. Future research will involve the determination of further investigation of antibacterial activity by using microdilution method, and the chemical characterization and structural elucidation of bioactive compounds.

4. Conclusions
The study of metabolite detection and the antibacterial activity of fungal endophytic extracts that living associated with T.crispa using the TLC-dot blot assay can be summarized that six endophytic fungi extracts showed strong antibacterial activity against both Gram-positive and negative bacteria. One extract had an excellent antibacterial activity (isolate No.6: TcBt1Bd-10). According to metabolites analysis using the TLC method along with TLC-Bioautography assay, the possibility of the chemical compounds that played a role in antibacterial activity of the extracts, including phenolics, flavonoids, alkaloids, and terpenoids. This study showed that fungal endophytic extracts associated with T.crispa might be used as promising sources of antibacterial. Further study needs to be done to isolate and purify the antibacterial compounds in the active extract and further investigation of antibacterial evaluation by using the microdilution method.

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Author Contributions
All authors wrote the manuscript and were equally working as main contributors.

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