Phytoconstituents Investigation on the Ethanolic Extract of *Azadirachta indica* var. Indonesian and Philippines

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Abstract. In the present study, phytochemical screening of ethanolic extracts of *Azadirachta indica* leaves from Indonesia and the Philippines revealed the presence of different phytoconstituents. Preliminary qualitative chemical test, TLC and LC-MS were used. TLC for all the extracts showed bands in long UV 366 for presence of flavonoid, tannin, saponin, terpenoid. As a result of LC-MS analysis of ethanolic extract *Azadirachta indica* leaves from Indonesia and Philippines, 10 compounds from Indonesian varian and 7 compounds from the Philippines varian were detected using m/z value. In conclusion, phytochemical screening based on TLC and LC-MS/MS show diverse bioactive compounds in ethanolic extract *Azadirachta indica* leaves from Indonesia and the Philippines. These can be effective approach for selecting best quality of varian leaves and planting area.

1. Introduction

Characterize natural product for new drug discovery has been concerned lately. Some of herbal products prove to offer synthetic drug substance. There are several factors that influence the quality of the herbs. Variation, environment conditions, storage, processing can be those influence quality factors of the herbs. Characterization compounds of herbs extract can be a standard procedure to find out the quality of the herbs.

*Azadirachta indica* (Neem) leaves are native of dry areas (Rojas-Sandoval *et al*., 2014). It was naturally distributed in Thailand, Malaysia, Philippines and Indonesia and has become one of the most widespread trees in tropical and subtropical areas. Neem plant as medicinal plant is reported to have antifungal (Lloyd *et al*., 2005), hepatoprotective (Pingale, 2010), anti-inflammatory (Jagadeesh *et al*., 2014), anthelmintic (Beltran *et al*., 2019), anti-cancer, insecticidal. The chemical constituents of *Azadirachta indica* leaves have a very important role in medicinal applications and it is believed due to its biologically active components.

In this present study, analytical method TLC and LC-MS was undertaken for identifying phytoconstituent. Thin layer Chromatography (TLC) is a very commonly used technique for identifying compounds, is a method of analysis in which the stationary phase, a finely divided solid, is spread as a thin layer on a rigid supporting plate; and the mobile phase, a liquid is allowed to migrate across the surface of the plate (Gennaro, 2000). This analytical tool is used because of its simplicity, speed of separation, cost effectiveness and high sensitivity.
LC-MS/MS are used for characterization and quantitation of herbal medicines because full characterization of these product. The advantages of LC-MS/MS are its high sensitivity and high-throughput to confirm the identity of the components in complex herbal extract, along with the detection and identification of unknown and unexpected compounds (Krug et al., 2008).

The aim of this research study was to assess the bioactive components present in the ethanolic extract of *Azadirachta indica* leaves varian Indonesia and Philippines using phytochemical screening and chromatographic analysis. So the result could be developed and applied to the pharmaceutical production and quality control of botanical product.

2. Material and Methods

2.1. Collection and Authentication of Plant Material

Fresh *Azadirachta indica* leaves were collected from Indonesia (Madura) dan Philippines (Camiling). The plant specimens were authenticated by Laboratorium of Plant Taxonomy, Universitas Brawijaya. Number identification of these plant is 0238/UN10.F09.42/03/2018.

2.2. Preparation of Plant Extract

The leaves were cleaned by washing with running water and shade dried and the milled to pass through 100-mesh sieve. The leaf powder was extracted by maceration for three days with 80% ethanol at room temperature. The extracts were concentrated at 45°C using Rotary vacuum evaporator to yield 80% hydroethanolic fraction as brownish green viscous residue. The concentrated extracts were keep in refrigerator at 4°C until further use.

2.3. Preliminary Phytochemical Screening

Test for the presence and absence of phytochemical compounds using standard methods involves the addition of an appropriate chemical agent to all the extract in a test tube and shaken. The different qualitative chemical test were performed for establishing profile of given extract for its chemical composition. Phytochemical screening of ethanolic extract of *Azadirachta indica* varian Indonesia and Philippines were carried out for alkaloids, flavonoid, saponin, tannin, terpenoid.

2.3.1. Alkaloid.

500 μL of ethanolic extract of *Azadirachta indica* varian Indonesia and Philippines of 10,000 ppm put in a test tube, then added 0.5 mL 2% hydrochloric acid. The solution was divided into three tubes. Tube 1 solution was added 0.5 mL acid solution dilute as a comparison, tube 2 was added 2-3 drops of Dragendorff’s reagent, and tube 3 was added 2-3 drops of Mayer’s reagents. If tube 2 was formed orange precipitation and tube 3 was formed yellowish precipitation. It indicated the presence of alkaloids.

2.3.2. Flavonoid.

500 μL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm put in a test tube, then added 1-2 ml hot methanol 50%. The solution was mixed by Magnesium and 4-5 drops of hydrochloric acid concentrate. Formation of red or orange colour indicated the presence of flavonoid.

2.3.3. Saponin.

500 μL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 10 ml distilled water and this was shaken 1 minute. Formation of bubble was added 2 drops of HCl 1 N. The stable bubble indicated the presence of saponin.

2.3.4. Tannin.

500 μL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 1-2 ml distilled water and this was added 2 drops FeCl₃ solution. Formation of green blackish colour indicated the presence of tannin.

2.3.5. Terpenoid.

500 μL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 0,5 ml chloroform and this was added 0,5 ml anhydrous acetic acid. Then 1-2 ml
concentrated sulphuric acid. The appearance of reddish brown or violet ring indicated the presence of terpenoids.

2.4. Thin Layer Chromatography
The presence of number of phytoconstituents flavonoids, saponins, tannins, terpenoid in ethanolic extract of *Azadirachta indica* var Indonesia and Philippines, which further became the basis for the phytochemical investigations through TLC on analytical plates over silica gel (TLC grade; Macherey-Nagel; Germany). The plates were dried in hot air oven at 110°C for 30 mins and then stored in a dry atmosphere and used whenever required. Samples were prepared by diluting the crude extracts of ethanol with respective solvent and then applied usually 1-10μl volumes to the origins of a TLC plate 1 cm above its bottom with the help of capillary tubes. After the application of the sample on the plate the plates were kept in TLC glass chamber (solvent saturated) than mobile phase was allowed to move through adsorbent phase up to top of the plate. The developed TLC plates were air dried. They were later spray with different spraying reagent. Rf were observed under long UV 366 nm. Calculation of Rf value is done with this formula:

$$Rf = \frac{\text{migration distance of substance}}{\text{migration distance of solvent front}}$$

2.5. LC-MS Screening
LC-MS screening was performed using LC-MS Thermo Scientific Dionex Ultimate 3000 RSL Cnano with micro flow meter in Lembaga Sentral Ilmu Hayati Universitas Brawijaya, Malang, equipped with Electrospray ionization (ESI). The LC-MS analytical data were optimized using a background subtraction technique of chromatography with the Analyst version: Compound Discoverer with mzCLoud MS/MS Library. The principle of this method is to reduce background. Data containing more real m/z were observed. Each compound was then identified from reference compounds based molecular weight of the structure.

3. Result and Discussion
3.1. Phytochemical Screening
The presence and absence of the phytochemical in *Azadirachta indica* var. Indonesia and Philippines are listed in the table 1. Our result revealed that flavonoid, tannin, saponin, terpenoids are present in ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines.

| Constituents  | Test performed                | Ethanolic extract |
|--------------|--------------------------------|-------------------|
|              | Indonesia | Philippines |
| Alkaloid     | Dragendorff’s Test          | -                  | -                  |
|              | Mayer’s Test                 | -                  | -                  |
| Flavonoid    | Hydrochloric acid Test       | +                  | +                  |
| Saponin      | Froth Test                   | +                  | +                  |
| Tannin       | Ferric chloride Test         | +                  | +                  |
| Terpenoids   | Salkowski's Test             | +                  | +                  |

3.2. Thin Layer Chromatography Profiling
TLC profiling of all extracts gives result the presence of number of phytochemicals. Various phytochemicals gives different Rf values. Different Rf values of the compound reflect an idea about their polarity. Mixture of solvents can be used for separation of pure compound from plant extract. The presence of any significant bioactive natural product indicates the necessity of separation of the compound from the
mixture of compounds through suitable chromatographic techniques. In the present study, flavonoids, saponin, tannin and terpenoid are confirmed to be present.

**Table 2.** Phytochemical analysis of *Azadirachta indica* leaves from Indonesia and the Philippines by Thin Layer Chromatography (TLC).

| Chemical Name | Solvent System | Extract Leaves | Neem Rf Values | Spray Reagent |
|---------------|----------------|----------------|----------------|---------------|
| Flavonoid     | ethyl acetate: methanol: aqua (5:1:5) | Indonesia | 0.03, 0.15 | Ammonia |
|               |                | The Philippines | 0.06, 0.77 |              |
| Saponin       | chloroform: methanol: aqua (13:7:2) | Indonesia | 0.26, 0.87 | Sulphuric acid 10% |
|               |                | The Philippines | 0.22, 0.85, 0.90 |              |
| Terpenoid     | toluene: ethyl acetate (7:3) | Indonesia | 0.19, 0.49 | Liebermann burchard |
|               |                | The Philippines | 0.27, 0.32 |              |
| Tanin         | n-butanol: acetic acid glacial: Aqua (2:0.5:1:1) | Indonesia | 0.27 | FeCl₃ 1% |
|               |                | The Philippines | 0.23 |              |

![Figure 1. Photograph of TLC of flavonoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).](image1)

![Figure 2. Photograph of TLC of saponin (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).](image2)

![Figure 3. Photograph of TLC of terpenoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).](image3)
Figure 4. Photograph of TLC of tanin (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).

3.3. LC-MS Screening

Figure 5. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

Figure 6. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Philippines leaves.

Table 3. Bioactive compounds in ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

| No. | Name of Compounds       | Group     | Molecular Formula | Molecular Weight | [M+HJ]+ (m/z) observed |
|-----|-------------------------|-----------|------------------|------------------|------------------------|
| 1.  | Betulin                 | Terpenoid | C_{30}H_{50}O_{2} | 442.728          | 442                    |
| 2.  | Ginsenoside             |           | C_{42}H_{72}O_{13} | 785.025          | 785                    |
| 3.  | Caryophyllene oxide     |           | C_{15}H_{24}O     | 220.356          | 220                    |
| 4.  | Soyasaponin I           | Saponin   | C_{46}H_{70}O_{18} | 943.134          | 943                    |
| 5.  | Ecgonine                | Alkaloid  | C_{9}H_{15}N_{3}  | 185.223          | 185                    |
| 6.  | Scutellarin             | Flavonoid | C_{31}H_{18}O_{12} | 462.363          | 462                    |
| 7.  | Epicatechin             |           | C_{18}H_{14}O_{6} | 290.271          | 290                    |
| 8.  | Icariin                 |           | C_{33}H_{40}O_{15} | 676.668          | 676                    |
| 9.  | Sesamolin               |           | C_{20}H_{18}O_{7} | 370.357          | 370                    |
| 10. | Lupeol                  |           | C_{30}H_{50}O     | 426.729          | 426                    |
Table 4. Bioactive compounds in 80% ethanolic extract of *Azadirachta indica* varian Philippines leaves.

| No. | Name of Compounds                  | Group       | Molecular Formula | Molecular Weight | [M+H]+ (m/z) observed |
|-----|-----------------------------------|-------------|-------------------|------------------|----------------------|
| 1.  | Betulin                           | Terpenoid   | C_{30}H_{50}O_{2} | 442.728          | 442                  |
| 2.  | Caryophyllene oxide               |             | C_{15}H_{24}O    | 220.356          | 220                  |
| 3.  | Andrographolide                   |             | C_{20}H_{30}O_{5} | 350.455          | 350                  |
| 4.  | 10-Deacetylbaccatin III           |             | C_{29}H_{36}O_{10} | 544.597         | 544                  |
| 5.  | 3-Acetyl-11-keto-β-boswellic acid|             | C_{32}H_{48}O_{5} | 512.731          | 512                  |
| 6.  | O-chloroaecetylcarbamoylfumigillo |             | C_{19}H_{28}ClN_{6}O_{6} | 401.884 | 401 |
| 7.  | Rutin                             | Flavonoid   | C_{27}H_{30}O_{16} | 610.521         | 610                  |

On the basis of the LC-MS the known compounds, 10 compounds (4 terpenoids, 1 alkaloid, 2 flavonoids, 2 lignins, 1 saponin) were identified from 80% ethanolic extract of *Azadirachta indica* varian Indonesia leaves and 7 compounds (6 terpenoids, 1 flavonoid) were identified from 80% ethanolic extract of *Azadirachta indica* varian Philippines leaves. Betulin and caryophyllene oxide were identified in both varian.

4. Conclusion
In the present study, ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines showed the presence of bioactive compound such as flavonoids, terpenoids, saponins, tannin. This study also leads to the further research in the way of isolation and identification of the active compound from the leave of *Azadirachta indica* var. Indonesia and Philippines using chromatographic and spectroscopic techniques.

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