Extraction and identification of Mitragynine from the Kratom Leaf (*Mitragyna speciosa*) using HFC-134a subcritical system

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Abstract. Kratom (*Mitragyna speciosa*) is a tree that useful as herbal medicine commonly found in Indonesia and other Southeast Asia Countries. Kratom has been consumed by chewing it or brewed like tea, it is useful to treated fever, muscle ache and diarrhea pain. Sedatives and stimulant effect on the central nervous system are side effect of Kratom leaves infusion. It contains mitragynine that have strong affinity on opioid receptor, therefor classified as new psychoactive substances (NPS). Others compounds in Kratom is 7-hydroxymitragynine, paynantheine, speciogy nine and speciociliatine. The aim of this study was to study the mitragynine and other secondary metabolites on the Kratom using subcritical HFC R134a extraction method and identified with TLC, LCMS/MS and GCMS. The HFC-134a subcritical system method is based on the advantage strength and physical properties of refrigerant R134a as solvent. Subcritical fluid extraction has potentially alternative method to isolate bioactive compound of the herbal plants. The result was 0.7878 g extract Kratom was produced, TLC spot at Rf 0.49, GCMS peak at RT at 18.327 and LCMS/MS peak of RT 7.12 with mass spectrum at 399.2m/z, 174.1m/z and 159.1m/z belong to mitragynine.

1. Introduction

Kratom (*Mitragyna speciosa*) is a tree indigenous Southeast Asia, one of them is Indonesia [1] especially grow in Putusibau, West Borneo [2]. The Kratom commonly used by way of chewing it, brewed like tea even made as cigarette to local society [3]. The Kratom leaf used as remedies such as, fever, relieve muscle pain, reduce appetite and diarrhea [1,4]. In Malaysia antiseptic substance from Kratom was used to treat postpartum recovery. Herbal medicine considered more safer for consumption than synthetic chemical-based medicine. Some terms usually used to mention Kratom as herbal medicine composition were Krypton, K2 or herbs and spices [5].

The studies on pharmacological effects of Kratom have been conducted such as analgesic activity, stimulant, anti-inflammatory, antioxidant, antinociceptive, antibacterial, and antidepressants [6-10]. The Kratom was known having ability as stimulant in low dose [11] and sedative effect in high dose.
Potential of sedative effect from ethanolic extraction reported bigger than those of diazepam, i.e. 27.9 mg/20 g BW [12], these effect mimic the act of opium which works suppress pain and used to reduce opioid with drawl syndrome [13], while depressant effect and stimulant in the center nervous system caused from side effect of Kratom [12]. This effect was mediated through monoaminergic receptors and opioid [14].

The Kratom leaf or the Puric leaf has been reported containing some alkaloid compounds i.e mitragynine 66.2%, paynantheine 8.6%, speciogynine 6.6%, 7-hydroxymitragynine 2%, and speciociliatine 0.8% [11].

Further studies are required to optimize secondary metabolite contents from The Kratom leaf. Therefore, in this study was conducted the extraction Kratom using subcritical HFC R134a extraction method. The method has been reported by Lelono et al [15] on the isolation of artemisinin from Artemisia annua, which is proven more effective than convention extraction method. This method has advantage of unique strength and physical properties from pure substance or mixed at critical pressure and temperature in equilibrium phase. Nowadays R134a refrigerant would be used as solvent in natural product extraction process. Because R134a has good strength, non-toxic, non-flammable, relatively stable, environmentally friendly, high purities and solubility and recovered solvent [16].

Tetrafluoroethane (HFC 134a) is safe to use in extraction technology. It works using low pressure and temperature. Extraction is possible using room temperature, lower temperatures but also higher temperatures. R134a is a non-polar fluorocarbon-based solvent that reaches a supercritical state at
101.2°C and 40.6 bar (compared to 31.0°C and 83.3 bar for carbon dioxide, CO₂) [17]. In the application, the use of HFC134a-based extractors has many advantages compared to other extraction methods, including: fast process; high final yield of the extraction (> 85%), low level of solvent wasted; ease of capacity expansion, and lower extraction costs compared to other extraction methods [15].

2. Materials and Methods

2.1. Materials

2.1.1. Plant material. The shredded and powdered dried leaf of Kratom (Mitragyna speciosa) were provided by UPT. KPHL GERBANG BARITO UNIT IX, The Forestry Service, Central Kalimantan, Indonesia.

2.1.2. Reagents and chemicals. N-hexane, ethyl acetate, methanol from Merck (p.a), LCMS/MS reagents grade from Merck, GCMS reagents grade from Merck, Sulfuric acid 5%, Refrigerant R134a Dupont (USA) as refrigerant (solvent), silica gel.

2.1.3. Apparatus. HFC R134a extractor unit, evaporator, column chromatography, silica gel 60 F254 TLC plates from Merck, GC-MS Agilent 7890B from Agilent, LC-MS/MS Waters Xevo G2-XS QTof from Agilent, UV-Vis lamp, Cimarex hotplate and Camag TLC chamber.

2.2. Method

2.2.1. Sample preparation. Dried leaves of Kratom were shredded and powdered to increase surface area to optimize result of extract product. Powdered sample was weighed (500 g). Sample was placed into both vessel of extractor, 250 g sample per vessel.

2.2.2. Extraction. Sample was extracted using HFC R134a extractor. Capacity was 5 liters, consist of two reactor extract chambers, collector extract chamber, pressure vacuum pump and chiller, both of reactor extract chamber must be cooled to 20 °C about 10 minutes prior to extraction. Releasing (HFC 134a) tube and inlet reactor extract chamber were turned (anticlockwise) to open it, pressure vacuum pump was pushed to flow about 2.5 kg HFC-134a into one of reactor chamber. Temperature, pressure and time were recorded as initial time of extraction process. Extraction was conducted for 3 cycles with 1 hour of extraction per cycle. To obtain the product, releasing the solvent out extract into collector extract chamber, which was heated up to 30°C. Extract was dried using air blower in room temperature. Extract was identified using Thin layer Chromatography (TLC), Gas Chromatography-Mass Spectrometry (GCMS) and Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS).

2.2.3. Thin Layer Chromatography (TLC). TLC silica gel 60 F254 were used for separation. Mobile phase was n-hexane : ethyl acetate solution (9:1). Approximately 1 mg sample was diluted in 1 mL methanol was applied as bands. Chromatograms were developed to 4/5 of the plate height, then dried, examined under UV lamp (254 nm).

2.2.4. Gas Chromatography-Mass Spectrometry (GCMS). Preparation for GCMS analysis was required about 10 mg extract in 1 mL chloroform and diluted. Sample was filtered prior for injection. Sample was injected for 1 μL using autosampler at 270°C with increased level temperature 10°C/minutes to 300°C. Column’s flow rate was 1 mL/minutes.
2.2.5. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS).
Approximately 10 mg sample was diluted in 1 mL methanol. Total of 5 μL was injected to Waters Xevo G2-XS QTof, equipped with ESI Source, pump and Masslynx PC Workstation. Qtop with positive ionization mode referred as Mass Spectrometry (MS). Parameter ESI that used involved capillary temperature 120°C and atomizer gas 5L/hours, voltage 2 kV. Full scan module from m/z 50-1200. Eluent was used H2O (A) and acetonitrile (B) grade LC-MS. Eluent’s flow rate was 0,3 mL/minutes.

3. Results and Discussions
The extraction was conducted three times at one hours of extraction time to maximize the yield content as compared to the studies reported on extraction of artemisinin using HFC-134a subcritical system in Lelono et al [15]. HFC-134a Subcritical extraction of Kratom has brown paste extract yielding 0.7878g from 500 gram of Kratom leaves, shown in figure 2. It was analyze using TLC, LCMS/MS and GCMS analysis method.

Figure 2. Kratom HFC-134a extract

Figure 3. TLC Chromatogram of Kratom extract

Thin Layer Chromatography was conducted as preliminary assay since it is easy, quick and cheap method to identified samples. Chromatogram of Rf sample compared with Rf standard of sample [18]. This method was prepared with gradient eluent n-hexane : ethyl acetate (9:1). Chromatogram was examined under UV lamp (254 nm). Mitragynine was spotted in Rf 0.48 shown in figure 3. According to study that conducted by Kowalczuk AP et al [19] Rf 0.49 was mitragynine.

Qualitative test of Kratom HFC-134a extract was conducted using GCMS and LCMS/MS. This was preliminaries step to provide an overview about compounds in Kratom HFC-134a extract. Elsa et al [20] have identified active compounds in Kratom with GCMS that mitragynine was separated at retention time (RT) 18.327 that shown in figure 4. Using the database, it was confirmed that peak at RT 18.327 was mitragynine.

Table 1. List of secondary metabolites compounds in Kratom HFC-134a extract from LCMS/MS data.

| No | Name Compounds         | Mass Fragment (m/z) | Retention Time (min) |
|----|------------------------|---------------------|----------------------|
| 1  | Mitragynine            | 399.220             | 7.12                 |
| 2  | 7-hidroxymitragynine   | 415.215             | 7.53                 |
| 3  | Isorhynchophylline     | 385.204             | 6.12                 |
| 4  | 3-Tert-butyl-4-methoxyphenol | 181.115    | 8.94                 |
Kratom HFC-134a extract was identified used LCMS/MS. Mitragynine showed retention peak at RT 7.12 minutes with mass fragment m/z 399.220 show in Figure 5 and others secondary metabolites compounds was also identified, that shown in Table 1. This is the first report of the extraction of myrtagynine using HFC-134a subcritical system. Kratom contains a number of indole alkaloids that are believed to be the primary contributors to its psychoactive effects. Chief among these is mitragynine, which typically constitutes 1–2% of the dry leaf mass and up to approximately two-thirds of the total alkaloid content [21]. Mitragynine was appeared at RT 7.12 with fragment m/z 399.2, 174.1 and 159.1 [22]. Mitragynine is alkaloid group, naturally synthesized from some organism including animal, plant, bacteria and fungi. Mitragynine (C_{23}H_{30}N_{2}O_{4}) have molecular weigh 389.5 g/mol, the most interested to study from The Kratom leaf is sedative effect and stimulant causing who consumed it. Mitragynine is reported as alkaloid compound of Kratom be expected have opioid effect 13 times higher than morphine [14]. Other metabolites which also interesting in Kratom is 7-hydroxymytraginine, which was reported presence in the Kratom HFC-134a extract.
Figure 5. LCMS/MS chromatogram of extract Kratom, mitragynine peak at RT 7.12 (A) and LCMS/MS mass chromatogram of extract Kratom, mass fragment of mitragynine at 399.220 m/z (B).

4. Conclusion
Extraction of Kratom leaves using HFC-134a give 0.15% of extract from dry leaves. The chromatography analysis of TLC Rf 0.48, GC-MS at RT 18.327 and LCMS/MS at RT 7.12 and mass fragment 399.220 m/z confirmed that was mitragynine is presence in the Kratom HFC-134a extract. Subcritical HFC R134a extraction method have good ability and efficient for extract natural plants. This is the first report of extraction of mytragynine from Kratom using HFC-134a subcritical system.

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