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Phytochemical Evaluation of Ethanol Extract *of Mitragyna S Peciosa:* Kidney, Liver and Delta-Opioid Receptor Expression in Rats

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KEYWORDS

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ABSTRACT:

Effect phytochemistry extract ethanol *Mitragyna speciosa* (kratom) was evaluated, with focus on its effects on the kidneys, liver, and expression delta-opioid receptor (DOR) in mice. Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS) identifies mitragynine as the dominant alkaloid (46.47 ± 0.004 mg/g extract), together with with betaine, dibenzylamine, and sterols. Analysis histopathology disclose degeneration dose -dependent tubular necrosis, necrosis, and infiltration inflammation of the kidneys, together with with Liver sinusoidal disorders and cholangitis. Immunohistochemical staining show altered DOR expression in the cerebrum and cerebellum, correlated with partial opioid agonist activity mitragynine. Findings This highlight potential therapeutic and risk kratom organ lesion, which emphasizes the need optimization dosage and assessment security term long .

1. Introduction

Diversity resource The nature found in Kalimantan makes local communities in general traditional has use leaf plant This as a stimulant at doses low For increase productivity work , relieve painful as well as overcome fatigue and as an analgesic to sedative at doses high , even in western countries generally used as a natural alternative to synthetic opioids especially For management painful chronic and symptomatic separated opioid substances . Plants This is *Mitragyna speciose* is known with kratom. Along with increasing interest to treatment herbal and alternative based, kratom has also become subject study scientific that has conducted by researchers, especially Because own content compound unique and potential active affecting the nervous system center and body organs. Kratom plants are abundant found in Southeast Asia especially Thailand, Malaysia and Indonesia. Complexity compound bioactive especially alkaloids such as mitragynine and 7 hydroxymitragynine cause challenge in understand mechanism work and its risks in a way comprehensive.

Contents alkaloid compounds that can interact with mu opioid receptors (MOR) and delta opioid receptors (DOR) in the brain this is what triggered it analgesic and euphoric effects , as well as α2 adrenergic receptors and serotonin 5-HT2A which affect atmosphere heart (Robayo Avendaño et al., 2021) . On studies pharmacokinetics also showed that alkaloid compounds contained in kratom can metabolized in the liver through CYP3A enzyme so that can increase potential opioid effects up to 5- fold (Smith et al., 2024). Although Thus, variations the alkaloid profile of each strain can cause difference effect significant pharmacological effects (Smith et al., 2024). The dominant alkaloid compounds in kratom are mitragynine, while 7 hydroxymitragynine is minor compounds.

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The mechanism of action of kratom in silico has been Lots done the main thing for know toxicity, as well as its potential against target proteins in the body human beings who will later can used as base in study in vivo. Toxicology studies show that change histopathology of the kidney and liver covering infiltration cell inflammation, degeneration until necrosis become reject measure it . While that , expression kratom compounds on the brain with show involvement in neurotransmitter modulation that can affectfunction cognitive and motoric. Interaction compound active kratom with enzymes and transporters in these organs will become attention special as security use of kratom in long term . Therefore that , although own activity therapeutic , effect toxic the use of kratom must investigated more carry on through studies pharmacokinetics , expression receptors , and biomarkers of organ damage . In the study This done For know kratom toxicity in a number of dose to kidney and liver mouse as well as Kratom expression on the cerebrum and cerebellum in the brain .

2. Research methods

1. Extraction leaf *Mitragyna speciosa*

Mitragyna speciose leaves as much as 10,000g is dried at a temperature room for 10 days , finely ground become powder dry coarse (<1 mm) and extracted with 96% ethanol for 120 hours. Extract evaporated with rotary evaporator until become mass semi-solid colored chocolate old and stored at room temperature temperature –20°C before used analysis next . Extract the resulting thick that is as much as 15g.

2. Identification Mitragyna Speciosa Alkaloid Compounds Using the LCHRMS Method

LCHRMS analysis was conducted at the Service Unit Integrated Research Laboratory (UPT) Integrated Research Laboratory (LRT) Brawijaya University. Extract sample thick diluted in accordance with solvent ethanol 96%. Dilution done with a final volume of 1500 µl, then sample vortex with speed 2000 rpm less more for 2 minutes. Next sample spun down at 6000 rpm for not enough more than 2 minutes, then Take the supernatant and then filter it with use a 0.22 um syringe filter and insert to in vial. Sample in vial ready for entered to the autosampler then injected to Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS). LC-HRMS instrument used type Thermo Scientific Dionex Ultimate 3000 RSLCnano with microflow meter, uses solvents A= 0.1% Formic acid in Water and B= 0.1% Formic acid in Acetonitrile. Using analytical column Hypersil GOLD PFP 50 x 1 mm x 1.9 u particle size, with analytical flow rate 40 uL / min, Flow gradient Run time: 35 minutes, column oven: 35 C. High Resolution Mass Spectrometer used that is Thermo Scientific Q Exactive with Full scan at 70,000 Resolution, with data dependent MS2 at 17,500 Resolution and Run time 35 minutes, and polarity with positive. For read results identification using Compound Discoverer with mzCloud MS/MS Library.

3. Identification Metabolites Secondary

Testing alkaloid extract done with made concentration of 100 ppm in a volume of 2 mL . Next added 2 mL BCG solution 10 -4 and 2 mL solution buffer phosphate pH 4.7 then extracted with 3 mL chloroform and taken phase chloroform with used funnel separator Then added chloroform on pumpkin measuring until the volume reaches sign 10 mL limit . All sample Then measured absorbance use spectrophotometer on long 289 nm wave . Manufacturing standard curve caffeine done with dilution solution stock 1000 ppm becomes three concentration namely 60, 100 and 140 ppm in a volume of 2 mL 2 mL For Then added 2 mL BCG solution 10 -4 and 2 mL solution buffer phosphate pH 4.7 then extracted with 3 mL chloroform and taken phase chloroform with used funnel separator Then added chloroform on pumpkin measuring until the volume reaches sign 10 mL limit . Absorbance measured with spectrophotometer on long 289 nm wave . Absorbance value Caffeine standard then made curve standard For get the equation y = mx + c is used For count mark level alkaloid extract , where y is equated with mark absorbance sample extract , while x is level equivalent alkaloids with caffeine (mg/gr extract) as standard .

4. Treatment Mouse

Study This to use mouse winstar male 2 months old with weight 200 Grams. Rats who have weighed each done acclimation for 5 days with availability sufficient food and drink on each cage. Treatment shared into 2 groups that is group 1 is mice that were given treatment dose kratom 300mg/L and group 2 is group mouse

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Healthy as control. Giving dose kratom on each group for 5 days in the morning and evening. The mice were then dissected to remove their kidneys and livers to assess organ damage, too part brain for know expression antibodies delta opioid receptor.

5. Assess Organ Damage Kratom On Organs

Organ the mice used For analysis toxicity is kidney and liver Then saved in a pot containing formalin Then do coloring hematoxylin eosin (HE). Observation stock histopathology labial mucosa given treatment that is scoring damage kidney and liver at 5 LP (Field) View) is different with 400x magnification which is then averaged. Observation This use microscope light (Nikon Eclipse type Ei) with help Optilab SIGMA Microscope Camera connected on computer.

6. 4. Kratom Against Delta Opioid Receptor

Rat organs used is brain, then done coloring using the polyclonal Phospho-OPRD1 (Ser363) antibody (LS-thermo-PA540246) with the secondary antibody Goat anti-rabbit IgG (H+L) (LS-thermo-31460). Calculation amount Expression of delta opioid receptor antibodies in the brain use mark percent area (%) assisted Image-J application. Observation This use microscope light (Nikon Eclipse type Ei) with help Optilab SIGMA Microscope Camera connected to a computer.

3. RESEARCH RESULT

Table 1. Analysis results compound *Mitragyna speciosa* ethanol extract use LCHRMS method

o	Name	Formula	Calc. MW	RT [min]	Area (Max.)	mzCloud Best Match
1	Mitragynine	C23 H30 N2 O4	398.22011	4.745	1.07E+10	98.4
2	Mitragynine	C23 H30 N2 O4	398.22011	4.958	8.76E+09	97.8
3	Mitragynine	C23 H30 N2 O4	398.22011	4.11	8.74E+08	96.6
4	di(3-pyridyl) 5 -(tert-butyl) isophthalate	C22 H20 N2 O4	376.14241	5,079	2.23E+08	92.7
5	Betaine	C5 H11 N O2	117.07907	1.92	2.09E+08	98.9
6	4,4-Dimethylzymosterol	C29 H48 O	412.37002	2.412	1.94E+08	-
7	p- Cumate	C10 H12 O2	164.08374	1.203	1.34E+08	-
8	NP-007909	C13 H20 O3	224.14124	1.211	1.20E+08	64.7
9	Dibenzylamine	C14 H15 N	197.12032	4.206	1.06E+08	99.6
10	4α-Hydroxymethyl-4β-methyl-5α-cholesta-8,24-dien-3β- ol	C29 H48 O2	428.36516	1,967	6.88E+07	-
11	Methyl 19-methyl-2-oxoformosanan-16-carboxylate	C21 H24 N2 O4	368.1732	3,706	6.67E+07	83.6

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12	4α-Methyl- zymosterol	C28 H46 O	398.35417	1,769	5.47E+07	-
13	DL-Dipalmitoylphosphatidylcholine	C40 H80 N O8 P	733.56383	0.712	5.40E+07	98
14	4α-Methyl- zymosterol	C28 H46 O	398.35417	2.294	4.83E+07	-
15	4-oxo-5- phenylpentanoic acid	C11 H12 O3	192.07866	1.184	4.43E+07	61.2
16	Bis(3,5,5-trimethylhexyl) phthalate	C26 H42 O4	418.30788	1,865	3.52E+07	98.3
17	Stearamide	C18 H37 NO	283.28743	1,681	2.70E+07	97.6
18	Stearamide	C18 H37 NO	283.28743	1.164	2.56E+07	97.2
19	4-methyl-5-oxo-2-pentyl-2,5-dihydrofuran-3-carboxylic acid	C11 H16 O4	212.1048	1.188	2.42E+07	62.9
20	Diazepam-d8	C16 H5 [2]H8 Cl N2 O	292.12084	1,514	2.39E+07	95.3
21	Citrus	C10 H16 O	152.1201	1.184	2.20E+07	79.9
22	12-Oxo phytodienoic acid	C18 H28 O3	292.2036	1.211	2.05E+07	85
23	NP-001798	C16 H17 N O2	255.12557	1.136	2.02E+07	93.3
24	Cycloeucalenone	C30 H48 O	424.37013	1,544	1.88E+07	-
25	SB236057A	C33 H34 N4 O3	534.26286	1,827	1.78E+07	88.8
26	Penicillic acid	C8 H10 O4	170.05788	1.165	1.65E+07	63.7
27	(2S,3S)-2,3-Dihydroxy-2,3- dihydrobenzoate	C7 H8 O4	156.04228	1.172	1.62E+07	-
28	2-Amino-1,3,4-octadecanetriol	C18 H39 N O3	317.29262	5.209	1.58E+07	69.1
29	1-Piperideine-2-carboxylate	C6 H9 N O2	127.0634	1.165	1.55E+07	-

Table 2. Alkaloid Test Results Extract Ethanol *Mitragyna speciosa*

Concentration (ppm)	Sample Absorbance		Total Alkaloids (mg/g extract)	Mean	SD	Total Alkaloids (mg/g) ± SD
500	U1	0.119	46.47	46.47	0.004	46.47 ± 0.004

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	U2	0.123	46.48			
	U3	0.115	46.47			

high alkaloid content , namely 46.47 ± 0.004 mg/g extract , provides base strong For activity biological extract This . Alkaloids, especially mitragynine , known own characteristic antimicrobial and antioxidant .

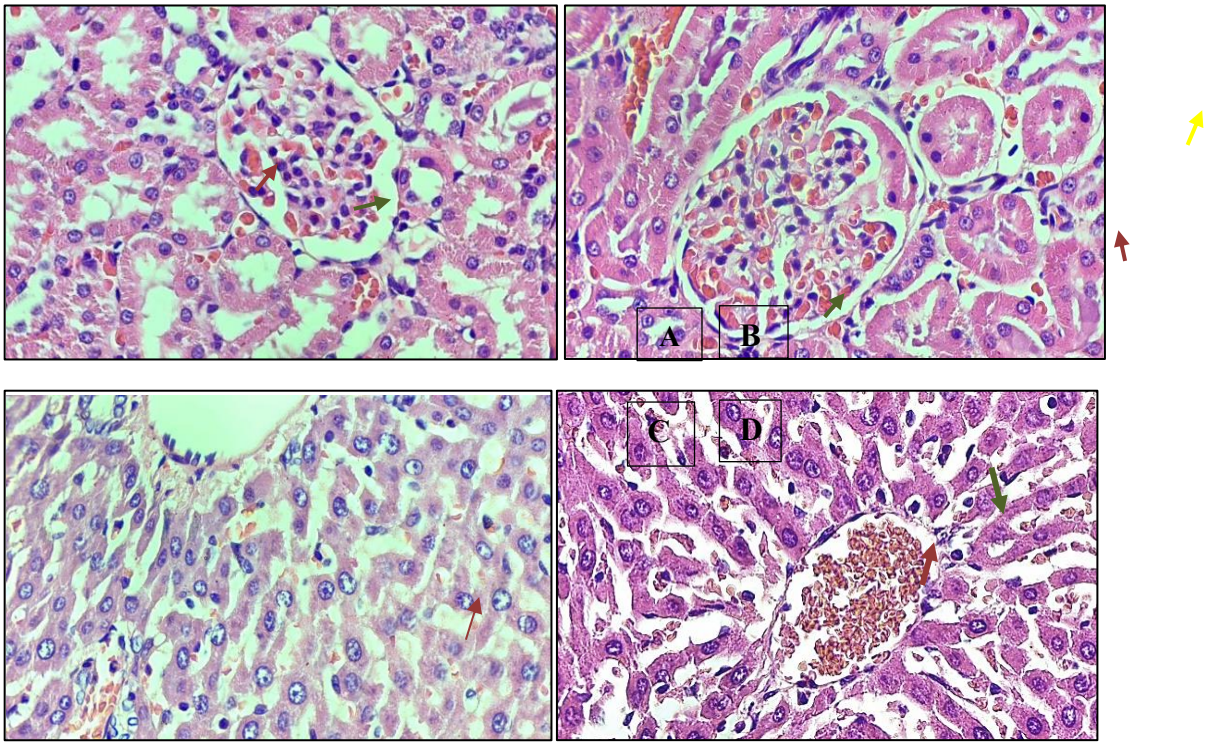

Figure 2. Histology kidney and liver (400x)
(A. Healthy Mice kidney, B. dose 300mg/L of Mice kidney, C. Healthy Mice Liver, D. dose 300mg/L of Mice liver)

→ : Endothelial : Healthy cells : Picnosis

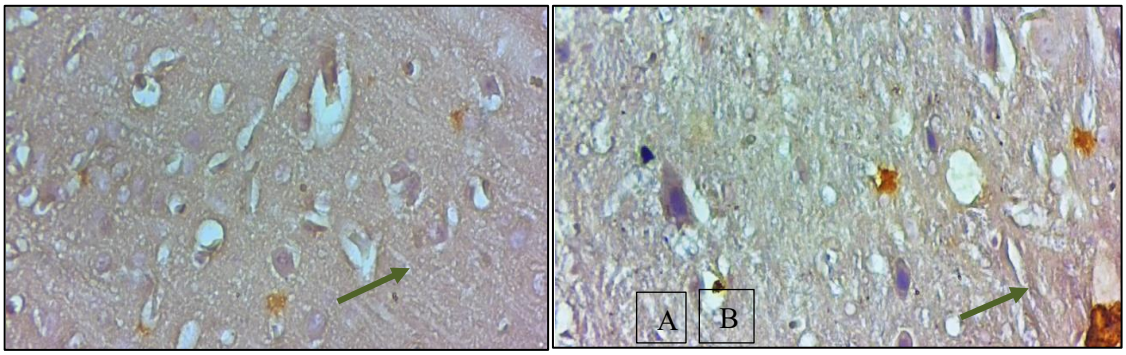

Figure 4. Immunohistochemistry of cerebrum and cerebellum (a. Healthy Mice, b. dose 300mg/L of mice brain)

4. Discussion

Identification alkaloid compounds in the extract ethanol leaf *Mitragyna speciosa* done use Liquid Chromatography-High Resolution Mass Spectrometry (LC–HRMS) instrument, where the use of LC-HRMS is for detect as well as identify compound bioactive in mixture complex like extract plants. Analysis results

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show existence various compound, with mitragynine as the main alkaloid detected in several time retention (RT) is different namely 4,745, 4,958, and 4.11 minutes with mz /Cloud Best Match respectively 98.4, 97.8, and 96.6 so own level compatibility high (Best Match >96%). In addition to mitragynine, other compounds such as betaine, dibenzylamine and several sterols were also identified organic acids. The LCHRMS method is in line with approach latest in analysis phytochemicals , where combination chromatography liquid and spectrometry mass resolution tall capable identify alkaloid compounds in general precision , even for isomers or compound with mass molecules that are almost The same (Lu et al., 2024) . Besides that , the use of column Hypersil GOLD PFP in research This own selectivity to polar and aromatic compounds because PFP column allows more separation Good For certain kratom alkaloids compared to with use column C18 as in the study (León et al., 2009) who use more C18 columns common and effective For non-polar compounds . However based on study (Kamble et al., 2019) which can identify minor compounds such as paynantheine, speciogynine and speciociliatine in extract kratom leaves . Differences results identification This Possible caused by sensitivity instruments, methods extraction, or variation in composition chemistry plants used in study this. So it is necessary alkaloid tests were carried out to ensure the presence and levels of alkaloids in the extract kratom leaves.

Alkaloid test was performed in a way quantitative with method spectrophotometry use BCG (Bromocresol Green) reagent and buffer phosphate pH 4.7, results analysis obtained show total alkaloid content of 46.47±0.004 mg/g extract. Use spectrometer mass resolution high (70,000) which allows more identification accurate based on mass and fragmentation of ions, so that with resolution tall This give level high accuracy in identify compound , thing the different with research conducted by (Kamble et al., 2019) which used spectrometer mass with resolution more low , so that limit ability For differentiate compound with very similar masses . Based on results research (table 2) shows that kratom alkaloids have potential powerful therapeutic, so kratom plants can become a natural analgesic alternative. Effects therapeutic from alkaloid activity can cause existence interaction with opioid receptors or hinder enzyme cyclooxygenase (COX). It is known that alkaloid types and concentrations certain can also give stimulant or sedative effects through adrenergic and serotoninergic receptors , but also have risk toxicity that is necessary beware (Eisenstein, 2019; Matsumoto et al., 2005; Tegeder & Geisslinger , 2004) . So it's necessary known activity Kratom compounds on kidney and liver organs as well as Kratom expression in the cerebrum and cerebellum of the brain to animal try mouse.

Analysis histopathology kidneys and liver in animals try , with observe existence change morphology covering degeneration , necrosis and infiltration cell inflammation of the tissue kidney as well as degeneration necrosis , cholingitis , infiltration and periportal bidding on the network heart (Meles et al., 2018; Murase et al., 2003) . Analysis results histopathology from study This show that giving dose in mice give influence on change histopathology kidney and liver shown with existence change structure tubules that can bother function filtration and reabsorption kidneys, as well as show existence congestion and hemorrhage indicating disturbance flow blood so that to aggravate condition hypoxia in the tissue. Then seems to happen too infiltration cell inflammation which is response from active immunity as reaction to damage network, which if in progress chronic can causes fibrosis and decreased function kidney term length. Histopathology results on the liver show happen changes in the structure of sinusoids and lobules heart that is not regular, thing the to signify existence damage network that can leading to disruption function heart. Change structure of the liver and kidneys the more looks with the more tall the dose given to the animal try mice. Then more carry on need done analysis immunohistochemistry to delta opioid receptor (DOR) expression in the cerebrum and cerebellum to his activities.

Expression of DOR depends on the dose and duration of administration. extract, in research This giving extract ethanol *Mitragyna speciosa* show existence change distribution and intensity expression receptor modulation. The expression looks Because existence interaction between compound mitragynine which is compound dominant in extract against DOR. Based on

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(Hassan et al., 2021; Liu et al., 2021; Lutz et al., 2014; Martini & Whistler, 2007) state that exposure opioid compounds can triggers downregulation or upregulation of DOR depending on the intensity and duration exposure , as well as condition pathological like inflammation show that compounds that have agonist activity causes effect analgesic but also triggers change expression Mitragynine receptor known own activity partial agonist to opioid receptors , which can stimulate change expression receptor as mechanism compensation or effect regulation feedback on delta-opioid receptor (DOR) which then trigger change expression receptor the in a way significant (Kruegel & Grundmann, 2018; Todd et al., 2020) . Change DOR expression can functioning as marker molecular toxicity or effect pharmacological. Therefore that, the DOR expression can made into as indicator important for evaluate effect neuropharmacological and neurotoxic from kratom extract. So that study this is, it is modulation observed DOR expression through immunohistochemistry can reflect effect pharmacodynamics direct consequence interaction said, good as adaptation protective and sign neurotoxicity early.

Based on from results overall in the research that has been done so use proper dosage and duration use kratom extract is needed done with careful, remember effect the therapy own risk toxic. So the research more advanced required For explore mechanism molecular toxicity, clinical trials security, as well as optimization formulation to minimize effect side with still maintain benefit therapeutic. With Thus, research This give runway scientific for development of kratom as analgesic natural, safe and controlled.

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