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Antiseizure Activity of Mitragyna inermis in the Pentylenetetrazol (PTZ) -Induced Seizure Model in Mice: Involvement of Flavonoids and Alkaloids

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Abstract
Ethnopharmacological relevance: Traditionally, *Mitragyna inermis,* is widely reported for its use in epilepsy management.
Aim of the study: This study aimed to investigate if *M. inermis* organic and aqueous extracts are able to control seizures induced by pentylenetetrazol (PTZ) on mice based on flavonoid fingerprints and alkaloidal contains.
Material and methods: Ethanolic extract and decoction-derived fractions from roots, leaves and stem were subjected to chromatographic fingerprinting using $AlCl_3$ and to the screening for their antiseizure effects using pentylenetetrazol (PTZ) -induced acute seizure model. From the fractions that showed potent bioactivities, the plausible antiseizure alkaloids were isolated by using thin layer chromatography and their structures were elucidated through ^{1}H NMR, 2D NMR, ^{13}C NMR and FAB-HR (*+ve* or *–ve*).
Results: All fractions, with the exception of DCM and hexane fractions, revealed remarkable flavonoid fingerprints. Acute PTZ-induced seizure test shows that ethanolic extract of stem bark (500 mg/kg b.w.), ethyl acetate extract of stem bark (500 mg/kg b.w.) and aqueous extract of leaves (300 mg/kg b.w.) significantly delayed the occurrence of hind limb tonic extension (HLTE), however, non-significant delay was observed in the onset of first myoclonic jerk (FMJ) compared to control animals. Isolation yielded four main alkaloids that are, pteropodine (**1**), isopteropodine (**2**), mitraphylline (**3**) and corynoxeine (**4**). Corynoxeine is a new compound from *M. inermis*.
Conclusion: This study suggests that flavonoid fingerprints are tracers of *Mitragyna inermis* anticonvulsant ingredients. Stem bark ethanolic and ethyl acetate extracts and leaf aqueous extracts contain anticonvulsant bioactive principles that delay notifying the hind limb tonic extension occurring in male NMRI mice. Furthermore, alkaloidal contains remain also the plausible bioactive anticonvulsant principles. All observations support the traditional use of *M. inermis* to manage epilepsy. However, further studies are needed to understand the effects of alkaloid fractions, flavonoids and the isolated compounds as a promising antiseizure agent derived from *M. inermis* in experimental animals.
Abbreviations
GABA, γ-aminobutyric acid; AEDs, Anti-epileptic drugs; PTZ, Pentylenetetrazol; DCM, dichloromethane; EtOAc, ethyl acetate; But, butanol; Ac, acetone; Aq, aqueous; EtOH, ethanol; $AlCl_3$, aluminum trichloride; HLTE, Hind Limb Tonic Extension; b.w., body weight; i.p., intraperitoneal; TLC, Thin Layer Chromatography; FMJ, first myoclonic jerk.
Key words: *Mitragyna inermis*, antiseizures, flavonoids, alkaloids, corynoxeine.

1. Introduction

Epilepsy is neurological and seems to be systemic disorder (Yuen et al., 2018). Epilepsy presents as partial or generalized seizures along with psychiatric comorbidities (Banerjee et al., 2009). Furthermore, people suffering epilepsy are more represented in the middle and low-income countries due to the enormous treatment gaps (Moshé et al., 2015). Social stigma, discrimination and misunderstanding are some of prejudices to live with epilepsy (Moshé et al., 2015). In the recent development of various antiseizure drugs (AEDs), some prevents seizures by acting through multiple ways such as acting on sodium channels (valproic acid, phenytoin, carbamazepine), calcium channels (ethosuximide), AMPA receptors (parampanel), GABA receptors (diazepam), or modulate the release of GABA (Gabapentin, pregabalin or valproic acid) (Macdonald, 1989; Kobayashi et al., 2019). Unfortunately, some of them still exhibit adverse effects like hypersensitivity reactions, mood changes, hepatotoxicity and are also ineffective against drug-resistant seizures (Copmans et al., 2018; Devinsky et al., 2018). Whereas, the primary goal of antiseizure drug therapy is complete freedom from seizures without adverse side effects (Shorvon et al., 2009). So, the needs to develop new antiseizure drugs become imperative (Moshé et al., 2015). Pentylenetetrazol causes seizures on its administration by preventing inhibitory effects of $GABA_A$ receptor (Huang et al., 2001). Thus, this GABA receptor antagonist is widely used as a model for antiseizure drug research. Natural sources like plants are suitable for development of various new drug candidates (Cragg et al., 1997).

Mitragyna inermis (Willd) O. Kuntze (*Rubiaceae*) is bushy tree or shrub (Arbonnier, 2002). In Burkina Faso, its leaves, roots and stem barks are used in mental illness and epilepsy treatment (Nacoulma, 1996; Arbonnier, 2002). Different modes of preparation such as maceration, infusion, decoction and leaching with various accessible solvents such as water, hydroalcoholic and acetonic solutions are used to obtain traditional medicines (Ouédraogo et al., 2021).

Moreover, previous studies were demonstrated anticonvulsant properties of leaf methanolic extract (300-1200 mg/kg) and stem bark ethanolic and aqueous extracts (62.5-500 mg/kg) (Timothy et al., 2014; Atinga et al., 2015). Those studies were reported on a moderate dose of convulsant agent (PTZ 60 mg/kg) and the anticonvulsant extract constituents were not specifically reported from this plant (Atinga et al., 2015). Therefore, these authors reported flavonoids, tannins, alkaloids, anthraquinone, glycosides and terpenoids on these extracts. However, tube tests reported on these potent extracts are preliminary phytochemical screenings and can be confirmed or refuted by thin layer chromatography screenings (Ouédraogo et al., 2021). Also, extensive phytochemical report revealed that *M. inermis* contains polyphenols, triterpenoids, indole and oxindole alkaloids (Shellard and Sarpong, 1969, 1970; Toklo et al., 2020, Ouédraogo et al., 2023). It has been reported that flavonoids and alkaloids or medicinal plant containing flavonoids and alkaloids interact with $GABA_A$ receptors leading to anticonvulsant activities (Jäger et al., 2011; Zhu et al., 2014, Copman et al., 2018). Besides, several AEDs like diazepam, carbamazepine, lorazepam, midazolam, brivaracetam, piracetam, aniracetam, oxiracetam, pramiracetam, nefiracetam, nebracetam, fasoracetam and levetiracetam licensed or in clinical development have the common function like alkaloids (Shorvon et al., 2009). Nevertheless, the knowledge of the potential contribution of flavonoids and alkaloids patterns of *M. inermis* different part extracts on its therapeutic properties is unknown. In the drugs research based on traditional recipes, it is important to assay or highlight ubiquitous tracers of biological activity due to the synergistic effects(Yuan et al., 2017). Indeed, flavonoids are generally followed in the standardization of raw materials from traditional medicine and chromatographic fingerprinting can be used in the quality control of medicinal plant materials (Ouédraogo et al., 2021).

This study aimed to investigate if *M. inermis* organic and aqueous extracts are able to control seizures induced by pentylenetetrazol (PTZ) on mice based on flavonoid fingerprints and alkaloidal contains.

2. Material and methods

2.1. Phytochemical study

2.1.1. Plant materials and extract preparation

Based on a previous study, *Mitragyna inermis* parts (leaf, stem bark and root) samples were collected from areas Banfora, Dindérésso and Boromo over September and November (Ouédraogo et al., 2022). Voucher specimen (UNB 939) is deposited in the Herbarium of Université Nazi Boni. The collected samples were subjected to total phenolic compounds assays and antioxidant properties evaluation, which are sensitive to ecological factors (Ouédraogo et al., 2022). At the end of these analyses, three samples were considered by plant part. Based on their phenolic contents and remarkable antioxidant potential, three samples by plant part were used to form composite samples per part, i.e. 40+40+40= 120 g of stem bark, 40+40+40=120 g of root bark and 30+30+30=90 g of leaves. The composite samples were used for the different extractions, maintaining the ratio of plant matter. The different preparations have been made in relation to the traditional way of preparation and the reported from previous studies (Timothy et al., 2014; Ouédraogo et al., 2023).

Acetonic extraction: 90 g of leaves, 120 g of root bark and 120 g of stem bark were submitted to 10-fold 70% acetone (v/w) in the conditions 1 h 30 min and 1500 rpm at 37°C. Then, filtration and centrifugation at 3800 rpm/35 min/4°C were carried out. The supernatants solvent were evaporated at 45°C with rotary evaporator and stored at 4°C until their use (Checkouri et al., 2020). This extraction yielded 32.74%, 13.76%, 22.87% from leaf, stem and root respectively.

Aqueous decoction and fractionation: 500 g of each part of *M. inermis* were used for extraction. Thus, the extraction was done with 10-fold distilled water (v/w) 100°C/30 min (Ranilla et al., 2010). Filtrate pH was reduced to 3-4 then, submitted to fractionation successively with polarity increasing (hexane, dichloromethane (DCM), ethyl acetate to butanol) . The residual water and butanol were evaporated by freeze-drying and the others solvent at 45°C with rotary evaporator. This fractionation yielded 13.6%, 5.97% and 4% for the leaf's decoction, ethyl acetate and butanol fractions, respectively. Also, 2% for ethyl acetate fraction of stem bark and 3.10% for ethyl acetate fraction of roots. Extracts and fractions were stored at 4°C until their use (Silva et al., 2018).

Ethanol extraction: Stem bark powder 120 g was macerated for 24 hours with 1200 mL of ethanol (99%). So, the raw material was macerated twice (the second with the recovered solvent). Then, the solvent was evaporated at 45°C with rotary evaporator (2% yield) and stored at 4°C (Timothy et al., 2014).

2.1.2. Flavonoid fingerprinting, bioassay-guided fractionation and alkaloids isolation

Extracts and fractions of each plant part were dissolved in methanol and applied on F254 silica plate. Then, elution was carried out over 8 cm with ethyl acetate: acetic acid: methanol: water (10:1.6:0.6:1.5) and the plates were viewed at UV 365 nm and after spraying with aluminum chloride (Figures **1**, **2**&**3**) (Ouédraogo et al., 2023). This first phytochemical screening is part of the confirmation of the presence of different subgroups of flavonoids reported as potential bioactive of traditional drugs. Also, this screening allows to group the fractions according to the tracers that are flavonoids in a preliminary standardization guide. All extracts were submitted to anti PTZ-induced seizures assays.

Furthermore, 20 g of the aqueous decoction of the leaves and 10 g of the ethanolic extract of the stem barks that exhibited potent antiseizure effects in PTZ-induced seizures were used for alkaloid treatments. So, the extracts were treated with 10% acetic acid for 12 h. At the end of the time, filtration was carried out and the pH of the filtrate was adjusted to 10 followed by extraction with DCM three times. Then, the DCM fraction was concentrated in rotavapor and dissolved in 5% sulfuric acid followed by extraction with hexane to remove the non-alkaloids. The residual aqueous phase pH was increased to 10 using ammonia followed by a new extraction with DCM. This last DCM fraction was concentrated in rotavapor, dissolved in methanol and dried under the hood. Finally, DCM was used to recover the yellow-colored soluble compounds. This process yielded 2.4% alkaloid fraction from the leaves and 7.8% alkaloid fraction from the stem bark. Similar spots were observed on both fractions after applied on precoated F254 silica plate and eluted with DCM: acetone (9:1). Then, a preparative TLC with the same system yielded four compounds; **1** (10 mg) and **2** (10 mg) from the leaves and **3** (6 mg), **4** (5 mg) from the stem bark.

2.1.3. General methods

TLCs were performed on precoated Kieselgel 60 F254 (Merck) plates. Plates were developed using DCM-Acetone then UV 254 and 365 nm. The isolated compounds were subject to ^{1}H NMR, 2D NMR HMBC, HSQC, COSY and NOESY, ^{13}C NMR and FAB-MS (*+ve* or *–ve*) analyses. Bruker Avance Neo NMR spectrometers operating at 400 and 600 MHz were used to run 1D and 2D NMR spectra giving coupling constants (*J*) in Hz and chemical shifts (δ) in ppm relative to the residual CD_3OD signal with TopSpin.

2.1.4. Structure elucidation of pteropodine (1), isopteropodine (2), mitraphylline (3) and corynoxeine (4)

Elucidation was carried out by comparison of ^{1}H NMR, 2D NMR, ^{13}C NMR and FAB-HR (*+ve* or *–ve*) data with the reported data.

Pteropodine (1): Amorphous white powder: FAB-HR (*+ve*): *m/z* = 369.1826 $[M+H]^+$; [M ($C_{21}H_{24}O_4N_2$) +H] = 369.1826. ^{1}H NMR (400 MHz, CD_3OD): δ = 8.53 (brs , NH) , 2.34 (dd, *J* = 6.0 , 2.4 Hz , H-3) , 3.21 (dd, *J* = 8.2 , 7.0 Hz, H-5a) 2.40 (t, *J* = 4.3 Hz , H-5b) , 2.29 (dd, *J* = 3.2 , 8.2 Hz, H-6a) 2.00 (ddd, *J* = 12.9 , 7.9 , 1.5 Hz , H-6b) , 7.05 (td, *J* = 7.6 , 1.1 Hz , H-9) , 6.86 (d, *J* = 7.9 Hz , H-10) , 7.19 (td, *J* = 7.7 , 1.3 Hz , H-11) , 7.27 (dd, *J* = 7.5 , 1.2 Hz , H-12) , 1.55 (d, *J* = 7.6 Hz, H-14a) 1.33 (dd, *J* = 3.7 , 7.2 Hz , H-14b) , 2.37 (dd, *J* = 3.3 , 2.5 Hz, H-15) , 7.50 (s , H-17) ; 4.47 (dq, *J* = 12.4 , 6.1 Hz , H-19) , 1.59 (d, *J* = 4.6 Hz , H-20) , 2.32 (d, *J* = 2.3 Hz, H-21a), 3.31 (H-21b) , 3.57 (s, 3H, OCH_3) , 1.39 (d, *J* = 6.1 Hz , CH_3). ^{13}C NMR (600 MHz, CD_3OD) : δ = 183.36 (C-2) , 75.31 (C-3) , 55.91 (C-5) , 34.98 (C-6) , 57.76 (C-7) , 134.60 (C-8) , 123.79 (C-9) , 110.57 (C-10) , 129.19 (C-11) , 124.18 (C-12) , 142.78 (C-13) , 30.84 (C-14) , 32.31 (C-15) , 110.57 (C-16) , 156.83 (C-17) , 73.72 (C-19) , 39.29 (C-20) , 54.27 (C-21) , 51.67 (OCH_3) , 169.66 (C=O) , 18.95 (CH_3). IR (KBr): Vmax = 3320.57, 2943.98, 2832.23, 668.42 cm^{-1}. UV/UV-Visible (MeOH) A = 1.347 (218 nm), 1.652 (247 nm).

Isopteropodine (2): Amorphous white powder: FAB-HR (*+ve*): *m/z* = 369.1821 $[M+H]^+$; [M ($C_{21}H_{24}O_4N_2$) +H] = 369.1821. ^{1}H NMR (400 MHz, CD_3OD): δ = 8.54 (brs , NH) , 2.42 (dd, *J* = 4.2 , 2.8 Hz , H-3) , 3.25 (td, *J* = 8.7 , 2.7

Hz , H-5) , 2.29 (ddd, *J* = 12.4 , 9.6 , 2.5 Hz, H-6b) 1.96 (dt, *J* = 13.0 , 8.5 Hz , H-6b) , 7.01 (td, *J* = 7.6 , 1.1 Hz , H-9) , 6.88 (d, *J* = 7.7 Hz, H-10) , 7.18 (td, *J* = 7.7 , 1.3 Hz , H-11) , 7.27 (d, *J* = 6.7 Hz , H-12) , 1.47 (dt, *J* = 13.1 , 3.8 Hz, H-14a) 0.79 (q, *J* = 12.2 Hz , H-14b) , 2.44 (dd, *J* = 8.2 , 3.7 Hz , H-15) , 7.42 (s , H-17) , 4.32 (dq, *J* = 10.4 , 6.2 Hz , H-19) , 1.59 (ddd, *J* = 8.8 , 7.7 , 4.4 , 3.6 Hz , H-20) , 2.39 (dd, 3.6 , 8.5 Hz, H-21), 3.36 (dd, *J* = 12.1 , 2.1 Hz , H-21b) , 3.58 (s, 3H , OCH_3) , 1.40 (d, *J* = 6.24 Hz , CH_3). ^{13}C NMR (600 MHz, CD_3OD) : δ = 183.17 (C-2) , 72.77 (C-3) , 55.10 (C-5) , 35.36 (C-6) , 58.51 (C-7) , 135.09 (C-8) , 123.43 (C-9) , 110.83 (C-10) , 128.98 (C-11) , 125.47 (C-12) , 142.35 (C-13) , 31.41 (C-14) , 31.92 (C-15) , 110.97 (C-16) , 156.50 (C-17) , 73.64 (C-19) , 39.36 (C-20) , 54.50 (C-21) , 51.56 (OCH_3) , 169.40 (C=O) , 18.78 (CH_3). IR (KBr): Vmax = 3320.43, 2944.11, 2832.23 cm^{-1}. UV/UV-Visible (MeOH) A = 0.790 (214 nm), 0.815 (244 nm).

Mitraphylline (3): Amorphous white powder: FAB-HR (*+ve*): *m/z* = 369.1809 $[M+H]^+$; [M ($C_{21}H_{24}O_4N_2$) +H] = 369.1809. ^1H NMR (400 MHz, CD_3OD): δ = 8.50 (brs , NH) , 2.45 (d, *J* = 2.5 Hz , H-3) , 3.25 (d, *J* = 2.5 Hz, H-5a), 2.55 (d, *J* = 1.6 Hz , H-5b) , 2.06 (ddd, *J* = 13.1 , 8.1 , 1.4 Hz, H-6a), 2.34 (dd, *J* = 4.6 , 1.7 Hz , H-6b) , 7.06 (td, *J* = 7.6 , 1.0 Hz , H-9) , 6.88 (d, *J* = 7.7 Hz , H-10) , 7.21 (td, *J* = 7.7 , 1.2 Hz , H-11) , 7.29 (d, *J* = 7.6 Hz , H-12) , 2.25 (dt, *J* = 3.1 , 3.0 Hz, H-14a), 0.99 (q, *J* = 11.6 Hz , H-14b) , 2.09 (d, *J* = 1.4 Hz , H-15) , 7.44 (s , H-17) , 4.42 (dd, *J* = 6.5 , 3.5 Hz , H-19) , 1.92 (dd, *J* = 4.6 , 2.3 Hz , H-20) , 3.11 (d, *J* = 7.3 Hz, H-21), 1.90 (s; H-21) , 3.55 (s, 3H , OCH_3) , 1.11 (d, *J* = 6.6 Hz , CH_3). ^{13}C NMR (600 MHz, CD_3OD) : δ = 180.29 (C-2) , 75.56 (C-3) , 54.97 (C-5) , 35.48 (C-6) , 57.07 (C-7) , 134.71 (C-8) , 123.73 (C-9) , 110.56 (C-10) , 129.23 (C-11) , 124.14 (C-12) , 143.17 (C-13) , 30.72 (C-14) , 31.56 (C-15) , 108.17 (C-16) , 155.63 (C-17) , 75.43 (C-19) , 42.91 (C-20) , 54.92 (C-21) , 51.39 (OCH_3) , 168.91 (C=O) , 15.08 (CH_3). IR (KBr): Vmax = 3323.82, 2943.70, 2832.08, 1448.24, 1113.69 cm^{-1}. UV/UV-Visible (MeOH) A = 0.199 (230 nm), 0.303 (216 nm), 0.160 (243 nm), 0.104 (260 nm), 0.097 (267 nm).

Corynoxeine (4): Amorphous white powder: FAB-HR (*+ve*): *m/z* = 383.1955 $[M+H]^+$; [M ($C_{22}H_{26}O_4N_2$) +H] = 383.1955. ^1H NMR (400 MHz, CD_3OD): δ = 8.55 (NH) , 2.39 (d, *J* = 2.0 Hz , H-3) , 2.45 (d, *J* = 9.1 Hz, H-5a), 3.28 (H-5b) , 2.30 (m, H-6a) , 2.00 (d, *J* = 11.9 Hz , H-6b) , 7.02 (td, *J* = 7.6 , 1.1 Hz , H-9) , 6.87 (d, *J* = 7.7 Hz , H-10) , 7.18 (td, *J* = 7.7 , 1.3 Hz , H-11) , 7.43 (d, *J* = 8.8 Hz , H-12) , 1.88 (H-14) , 2.50 (d, *J* = 3.3 Hz , H-15) , 7.25 (s , H-17) , 5.49 (m , H-18) , 4.90 (d, *J* = 2.1 Hz , H-19) , 2.84 (d, *J* = 11.0 Hz , H-20) , 3.17 (dt, *J* = 11.0 , 3.6 Hz, H-21a) 1.95 (m , H-21b) , 3.55 (s , OCH_3) , 3.72 (s , OCH_3). ^{13}C NMR (600 MHz, CD_3OD) : δ = 180.26 (C-2) , 73.88 (C-3) , 55.02 (C-5) , 36.06 (C-6) , 58.12 -C-7) , 135.0 (C-8) , 123.45 (C-9) , 110.72 (C-10) , 128.86 (C-11) , 125.94 (C-12) , 142.3 (C-13) , 24.17 (C-14) , 73.37 (C-15) , 112.56 (C-16) , 161.48 (C-17) , 140.87 (C-18) , 115.97 (C-19) , 43.6 (C-20) , 60.05 (C-21) , 51.29 (OCH_3) , 61.85 (OCH_3) , 170.31 (C=O). IR (KBr): Vmax = 3320.28, 2943.33, 2832.29, 1448.15, 1114.24 cm^{-1}. UV/UV-Visible (MeOH) A = 0.987 (220 nm), 1.058 (230 nm).

Compounds **1**, **2** and **3** appeared as white amorphous powder with the respective molecular weights 368.1826, 368.1821 and 368.1809. All matched to the same calculated formula $C_{21}H_{24}O_4N_2$. In addition, the common fragment ions appeared at *m/z* = 339.3 $[M-30]^+$, 325.2 $[M-44]^+$, 291.2, 227.3, 164.1 $[M-205]^+$. Herein, they reveal similarity to pentacyclic oxindole alkaloids. On TLC with solvent system DCM: Acetone (9:1), these compounds showed different frontal reference high (**1** > **2** > **3**). All compounds (**1**, **2**, **3**) in ^1H NMR revealed presence of one methoxy group. That means, these compounds are the stereoisomers of pentacyclic oxindole alkaloids. Therefore, configuration assignment based on the chemical shift described by Beckett et al. showed that compound **1** and compound **2** are on β configuration with C19, C20 but α configuration with C15. Also, compound **1** (δd-CH_3 = 1.39) and compound **2** (δd-CH_3 = 1.40) revealed ***cis*** **D/E** ring junction with three-proton doublet for the methyl (Beckett et al., 1965). Compound **3** three-proton doublet appeared at 1.11 ppm as **D/E** ring ***trans*** junction (Beckett et al., 1965). Correlation was observed between H-17 doublet and the proton H-15 via a long range in compounds **1**, **2** and **3**. However, according to asymmetric centers and their NMR spectra assignment by theoretical calculations of shielding constants of Paradowska et al., compound **1** (***δ*C3** = 75.31) and compound **3** (***δ*C3** = 75.56) appeared as ***7R*** alkaloids, but compound **2** (***δ*C3** = 72.77) seemed like ***7S*** alkaloid. Also, compound **1** (***δ*C20** = 39.29) and compound **2** (***δ*C3** = 39.36) revealed to be ***20S*** alkaloid but ***20R*** alkaloid for compound **3** (***δ*C3** = 42.91) (Paradowska et al., 2008). For that, compound **1** (***7R***, ***20R***) *allo*-type isomer is identified to pteropodine (uncarine C), compound **2** (***7S***, ***20R***) also *allo*-type isomer is identified to isopteropodine (uncarine E) and compound **3** (***7R***, ***20R***) normal type isomer matched to mitraphylline (Toure et al., 1992; Paradowska et al., 2008; Giménez et al., 2010, Salim and Ahmad, 2010). Full ^1H and ^{13}C NMR signals assignment was made using HMBC, HSQC, COSY and NOESY correlation data. These compounds were previously reported from *M. inermis*.

Compound **4** appeared in molecular weight and formula 382.1955 and $C_{22}H_{26}O_4N_2$ respectively. Peaks at *m/z* = 339.2, 325.2 and 164.0 revealed the presence of olifinic and oxindole fragments. ^1H NMR revealed the presence of two methoxy groups at δH= 3.72, δC= 61.85 and δH= 3.55, δC= 51.29. Paradowska et al. theoretical calculations of shielding constants were used for oxindole alkaloids asymmetric centers and NMR spectrum assignment. So, Compound **4** (δC3 = 73.88; δC20 = 43.6) is (***7R, 20R***) alkaloid. The chemical shifts δC18 = 140.87, δH18 = 5.49 and δC19 = 115.97, δH19 = 4.90 are characteristic of the methylene group. It appeared to be a tetracyclic oxindol

alkaloid. However, ^{1}H NMR, 2D NMR, ^{13}C NMR and FAB-HR compound **4** matched to those of rhyncophylline 18, 19 didehydro like corynoxeine reported from *Mitragyna speciosa* (Paradowska et al., 2008; Flores-Bocanegra et al., 2020). Thus, in this study, we isolated new specific tetracyclic oxindole named corynoxeine from *M. inermis*.

2.2. Acute PTZ-induced seizure model in mice

All the procedures were performed according to the institutional animal care committee of the International Center for Chemical and Biological Sciences (ICCBS) (on January 7th, 2019, Animal study protocol was submitted to ICCBS/University of Karachi, Pakistan ethical committee and the application was accepted after revision on February 20th, 2019, protocol 2019-006). Seventy-six (76) male NMRI mice with weights between 20 and 24 g were chosen for the experiment. Mice were sourced from ICCBS animal research facility. An experiment was conducted in the light of the daylight/darkness cycle at 25±2ºC with appropriate moisture. Animals were brought into the experimental room 1 h prior to the start of the experiment for the acclimatization and separated into control and test groups. 110 mg/kg body weight (b.w.) PTZ as convulsive dose was selected based on an acute crisis model. Mice were grouped together in batches of seven (07) mice for each dose and control. After acclimatization, the controls were treated with distilled water 10 mL/kg b.w. by intraperitoneal (i.p.). After 30 min, PTZ was injected to the animals and the seizure main stages development were observed. The extract ability to attenuate seizure threshold is indicated by absence of seizures at least 5 seconds (Atinga et al., 2015). For the tests with the extracts and fractions of *M. inermis*, the seven mice for each dose were subdivided into two sub-groups of three (03) mice for the first testand (04) mice for the additional test. During the first phase, if no major capacity was observed to mitigate the effects of pentylenetetrazol at a given dose, the additional test was not carried out. The following doses prepared in distilled water were considered based on data in the literature (Timothy et al., 2014; Atinga et al., 2015):

- ✓ Aqueous decoctate of leaves: 500, 300 and 150 mg/kg b.w. ;
- ✓ Additional fractions acetone+ ethyl acetate + butanol of leaves: 500 and 250 mg/kg bw. ;
- ✓ Stem bark ethanolic extract: 500 and 250 mg/kg bw;
- ✓ Additional stem bark ethyl acetate + acetone fractions: 500 and 250 mg/kg b.w. ;
- ✓ Stem bark ethyl acetate fraction: 500 and 250 mg/kg bw;
- ✓ Stem bark acetone fraction: 500 and 250 mg/kg b.w. ;
- ✓ Additional root barks ethyl acetate + acetone fractions: 500 and 300 mg/kg bw.

For the test groups, the suitable dose of the extract was injected by i.p. to the animals, then after 30 min, a convulsive dose of PTZ was administered and the delay in the onset of first myoclonic jerk (FMJ) and hind limb tonic extension (HLTE) were observed and recorded for each animal. The flowchart of the whole study is presented in figure 4.

2.3. Statistical analysis

The statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparison using Graph Pad Prism - 8 Statistical Software Package at significance level set as $p < 0.05$.

3. Results and discussion

Antiseizures (AED) are more or less effective against generalized, myoclonic and absent tonic-colonial seizures (Macdonald, 1989). Indeed, around 30% of patients do not respond to the currently available AEDs (Moshé et al., 2015). In traditional medicine, *M. inermis* is used to manage epilepsy. Unfortunately, there was no data on the required levels of herbal materials for therapeutic value. Chromatographic fingerprinting is used in the quality control of medicinal plant materials (Ouédraogo et al., 2021). This report focused firstly on flavonoid fingerprints as tracers of bioactive contain in various fractions of *M. inermis*. As a result, DCM and hexane fraction didn't show the remarkable flavonoid fingerprints (**Figure 1, 2&3**). In the different parts, decoction, acetone, ethyl acetate and butanol fractions revealed various flavonoid fingerprints. Using flavonoid fingerprints, as fraction standardization, aqueous decoctate of leaves appeared atypical however, leaves ethyl acetate, butanol and acetone fractions appeared typical. Also, stem bark ethyl acetate and acetone fractions appeared typical, as well as root barks ethyl acetate and acetone fractions. Thus, for the same part, fractions may be combined for their similar flavonoid fingerprints. We noticed variability in flavonoids groups contain (**Figure 1, 2&3**). Other studies reported the presence of flavonoids in *M. inermis* parts but still on content analyses (Nacoulma, 1996; Timothy et al., 2014; Atinga et al., 2015, Ouédraogo et al., 2020).

Secondly, we conducted an investigation of *M. inermis* roots, leaves, and stem bark standardized fractions effects on acute PTZ-induced seizures in NMRI mice. All findings were presented in **Table I** and **Figure 6**. The preliminary study revealed that leaf decoction, stem bark ethyl acetate extract and ethanolic extract delayed the onset of PTZ-induced convulsions. In contrast, root fractions did not show antiseizure effects in the PTZ-induced seizure test in mice (**Table I**). The using of *M. inermis* root in the management of epilepsy has not been reported in any ethnobotanical survey. This lack of antiseizure properties might justify its non-utility in traditional medicine. Herbal

medicinal product must be manufactured from the indicated part of the plant and must be free of other parts of the same or other plants (Ouédraogo et al., 2021). The acetone stem bark fraction failed to demonstrated any effect, but the ethyl acetate stem bark fraction was found to be responsible of the combined antiseizure effects (**Table I)**. With the aim to confirm the abilities of *M. inermis*-derived fractions to reduce the seizures, more assays were carried out (**Figure 6**). PTZ 110 mg/kg b.w. showed FMJ at 49±0.67 sec and HLT at 65.33±4.22 sec in control group. So, ethyl acetate extract at 500 mg/kg b.w., decoction at 300 mg/kg b.w. and ethanol extract at 500 mg/kg b.w. revealed the noticeable delay of HLTE occurs (more than 10 min). Moreover, no significant antiseizure effect was observed between stem bark ethanolic extract (500 mg/kg b.w.) and leaf decoction (300 mg/kg b.w.) ($p > 0.05$). In contrast, all the fractions failed to significantly delay the onset of first myoclonic seizures. These results suggested that *M. inermis* delay the seizures, specifically delayed HLTE occurred, but failed to show the abilities to delay first myoclonic seizures. The lack of abilities to delay the onset of first myoclonic jerks suggests that *M. inermis* might require more time to exhibit antiseizures effects after the administration of the extract. However, the results observed in this study with the stem bark ethanolic extract are in agreement with the reported on temporary absence of seizures at 500 and 250 mg/kg b.w. (Timothy et al., 2014). Also, we reported that *M. inermis* leaves methanolic extract prevented seizures at the doses more than 600 mg/kg (Atinga et al., 2015). But this latter study employed high doses of extracts. In traditional drug preparation methanol is subjected to usage limits (Ouédraogo et al., 2021). The high dose of PTZ used in this study to induced seizure may justify variability of anticonvulsant properties and lack of remarkable latency to FMJ occurred compared to the reported study (Timothy et al., 2015; Atinga et al. 2015). It has been reported that flavonoids or medicinal plant extracts containing flavonoids interact with $GABA_A$ receptors leading to anticonvulsant activities (Jäger et al., 2011; Zhu et al., 2014, Copman et al., 2018). Indeed, the variability could arise from the difference of flavonoid fingerprinps observed with leaves and stem bark leading to the variation of the HLTE delay. Moreover, it may be suggested that these active extracts might act as agonist of $GABA_A$ receptor, similar to the effects other AEDs that prevent seizures in PTZ-induced seizure test (Shorvon et al., 2009, Kobayashi et al., 2019). So those derived fractions could be powerful sources of anticonvulsants. In addition, multiple studies reported lower toxicity of *M. inermis* different part-derived extracts in mice and rates (Monjanel-Mouterde et al., 2006; Ouedraogo, 2007; Timothy et al., 2014). Our latest research on these mixed fractions revealed that they are less hazardous to mouse fibroblast and human hepatocyte cell lines. (Ouédraogo et al., 2023). Flavonoids could therefore be tracers to guarantee the quality and therapeutic value of *M. inermis* parts preparation in traditional use and the research of anticonvulsant phytomedicines.

A thorough phytochemical analysis was carried out on the most anticonvulsant extracts as well as stem bark ethanolic extract and leaf decoctate. The fractionation revealed that the alkaloid fraction represented 2.4% of leaf decoction and 7.8% of stem bark ethanolic extract. Traore et al. reported that alkaloid-rich fraction showed low toxicity on monocyte proliferation through inhibition of mammary cell protein production but no mutagenic or genotoxic activities (Traore et al., 2000). Subsequently, in order to know these fraction alkaloid ingredients, TLC on the derived alkaloid fractions offered the main oxindole alkaloids viz pteropodine, isopteropodine and mitraphylline. In addition, we isolated the new tetracyclic oxindol alkaloid, corynoxeine (**Figure 5**). Moreover, pteropodine and isopteropodine were reported for their lack of adverse effects on cell culture and DNA (Lee et al., 1999; Aponte et al., 2009; Ahmad and Salim, 2015). Also, isopteropodine showed a neuroprotective effect without toxicity until 10^3 µM (Ahmad and Salim, 2015). However, *M. inermis* alkaloid content was already reported (Shellard and Sarpong, 1969, 1970; Toure et al., 2000). Alkaloid extract and compounds of other *Mitragyna* species as well as *Mitragyna speciosa* are reported to exhibit benefit pharmacological effects on the brain, but require moderate doses (Janie et al., 2010). Indeed, alkaloid such as mitragynine interact with neurons by blocking calcium channel (Farah et al., 2016). Therefore, neuronal Ca^{2+} channel-blocking effect abolish seizures (Kobayashi et al., 2019). Mitragynine is also reported in *M. inermis* leaf and stem bark (Shellard and Sarpong, 1969, 1970; Toure et al., 2000). However, indole alkaloids such as ibogaine similarly to pteropodine, isopteropodine and mitraphylline isolated in this study is reported to exhibit anticonvulsant activity by blocking N-methylD-aspartate receptors. Also, tetracyclic oxindole alkaloids such as rhynchophylline and isorhynchophylline present in *M. inermis* leave and stem bark have been reported with anticonvulsant activities by inhibiting N-methylD-aspartate receptors (Zhu et al., 2014). Thus, the abilities of decoction and ethanolic extracts to exhibit acute antiseizure effects in PTZ-induced seizure test might be attributed to the alkaloid fractions with the plausible synergistic action of pteropodine, isopteropodine, corynoxeine and mitraphylline or due to flavonoid contains. Some AEDs such as phenytoin, carbamazepine and diazepam exhibit alkaloid functions (Kobayashi et al., 2019). However, the pro-seizure effect reported with the ingestion of *Mitragyna speciosa* alkaloids calls for having moderate look and use of *Mitragyna inermis* alkaloids in the treatment of seizures.

4. Conclusion

This study suggest flavonoid fingerprints are tracers of *Mitragyna inermis* anticonvulsant ingredients. Stem bark ethanolic and ethyl acetate extracts and leaf aqueous extracts contain anticonvulsant bioactive principles that delay the hind limb tonic extension occurring in male NMRI mice. Furthermore, alkaloid contains remain also the plausible bioactive anticonvulsant principles. All observations support the traditional use of *M. inermis* to manage epilepsy. However, further studies might help to understand alkaloid fractions, flavonoids and the isolated compounds effects as a promising antiseizure agent derived from *M. inermis* in experimental animals.

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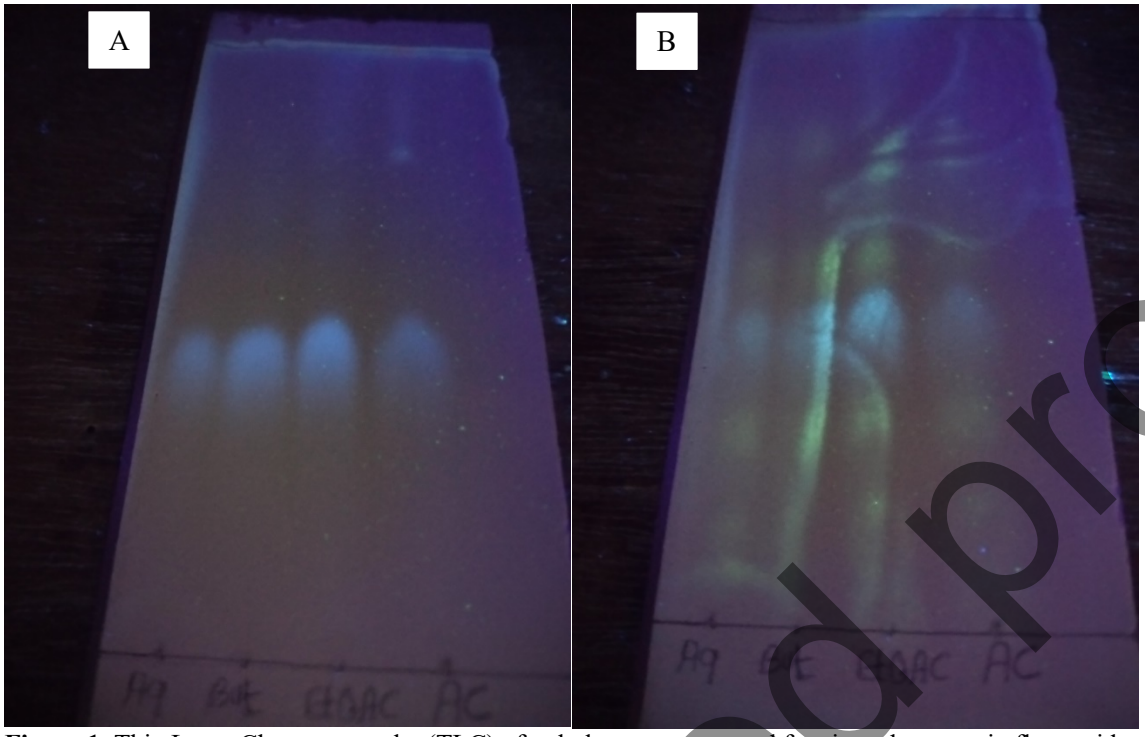


Figure 1: Thin Layer Chromatography (TLC) of only leaves extract and fractions that contain flavonoids, A: U.V. light; B: $AlCl_3$ spraying+U.V. light. **Aq**=aqueous, **But**=butanol, **EtOAc**=Ethyl acetate, **Ac**=Acetone fractions. Four additional yellow spots after spraying revealing flavonoids contain. Butanol, Ethyl acetate and Acetone appeared as same compound group contains.

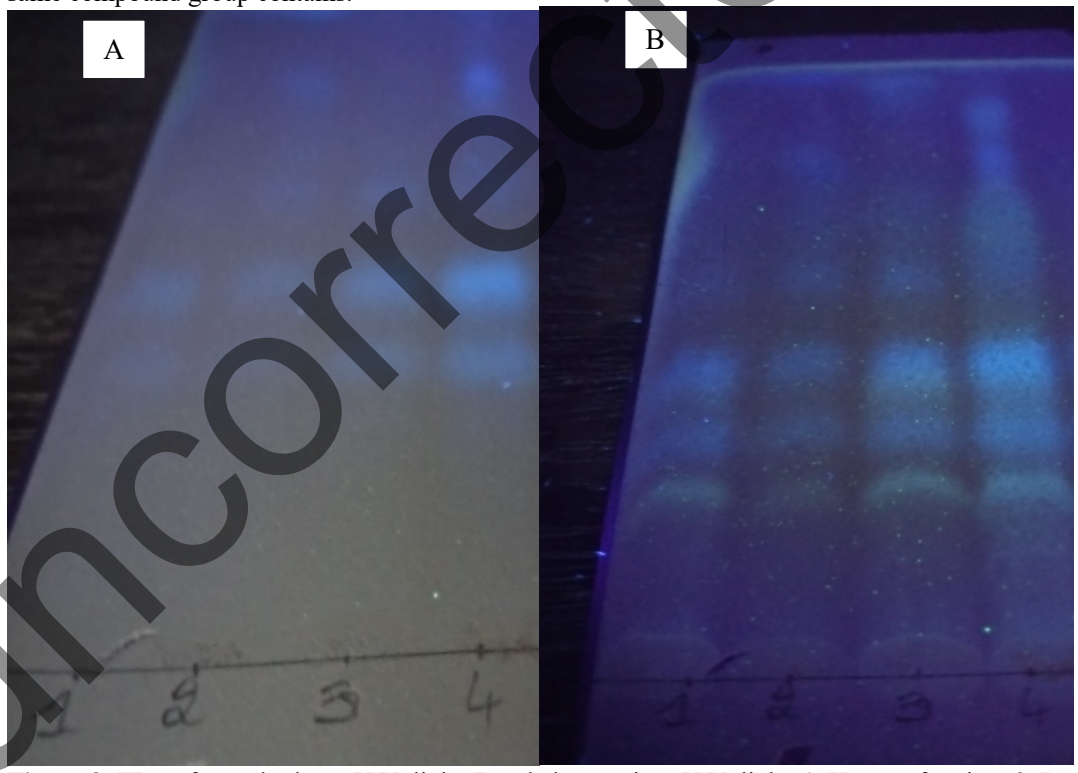


Figure 2: TLC of stem bark, A: U.V. light; B: $AlCl_3$ spraying+U.V. light. 1. Hexane fraction; 2. DCM fraction; 3. EtOAc fraction, 4. Acetone fraction. Three additional yellow spots after spraying revealing flavonoid contains. Most present in 3 and 4 that looked same.

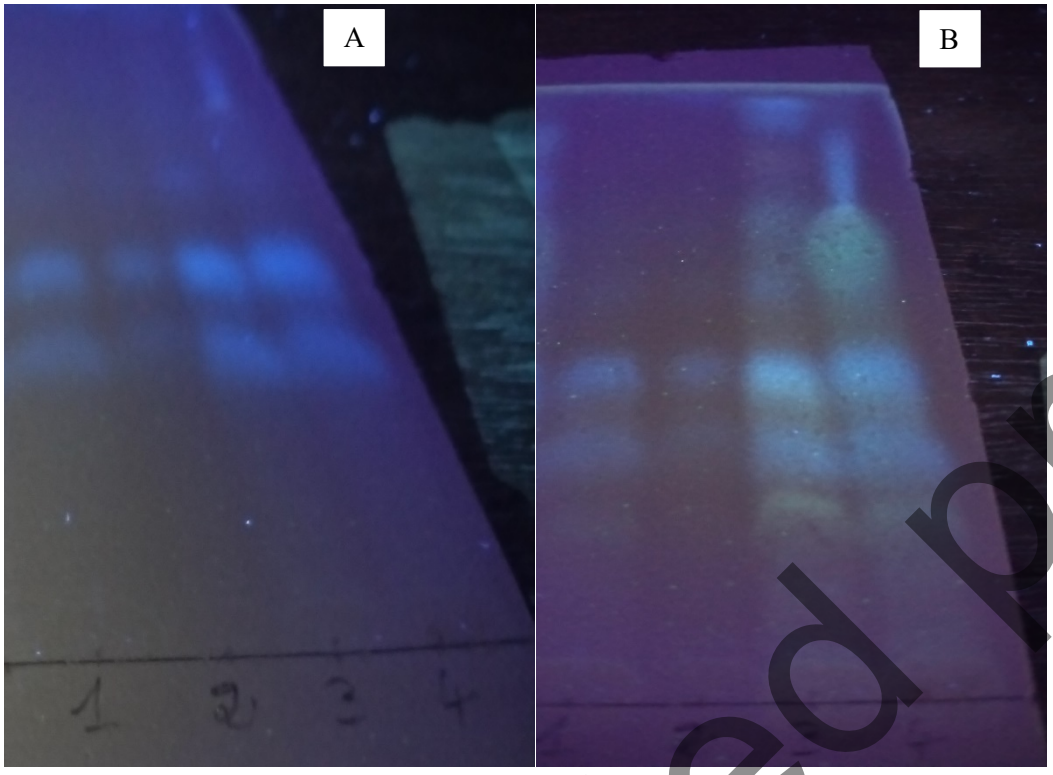
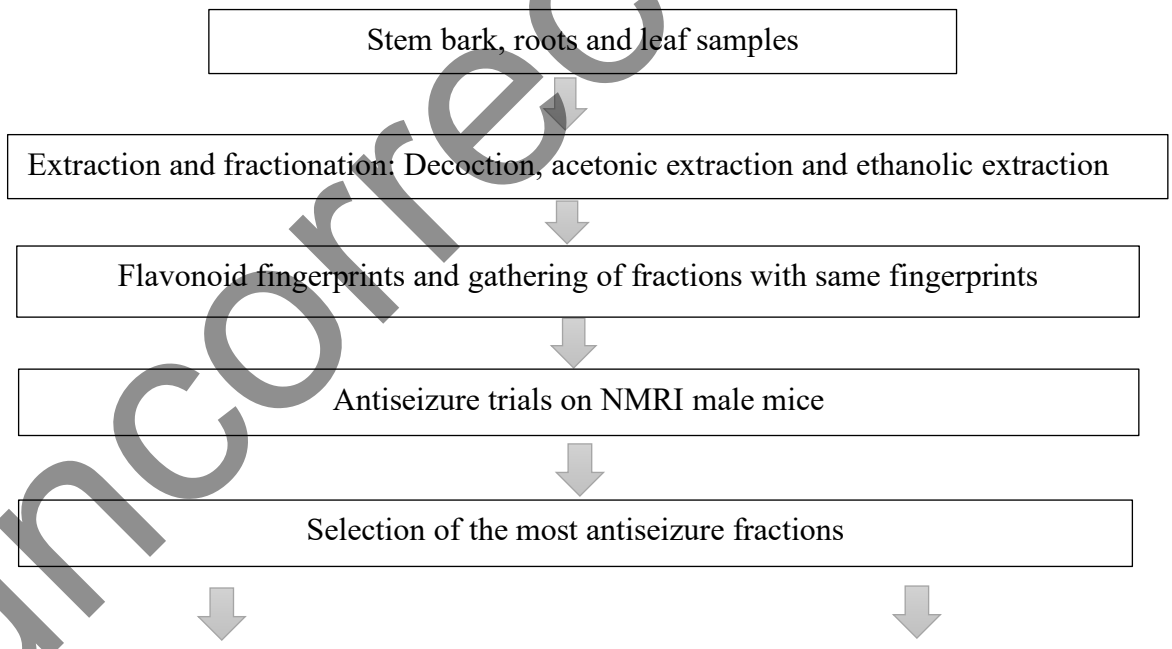


Figure 3: TLC of roots, A: U.V. light; B: $AlCl_3$ spraying+U. V. light. 1. Hexane fraction; 2. Dichloromethane (DCM) fraction; 3. Ethyl Acetate fraction, 4. Acetone fraction. Three additional yellow spots after spraying revealing flavonoid contains. Most present in 3 and 4 that appeared same.



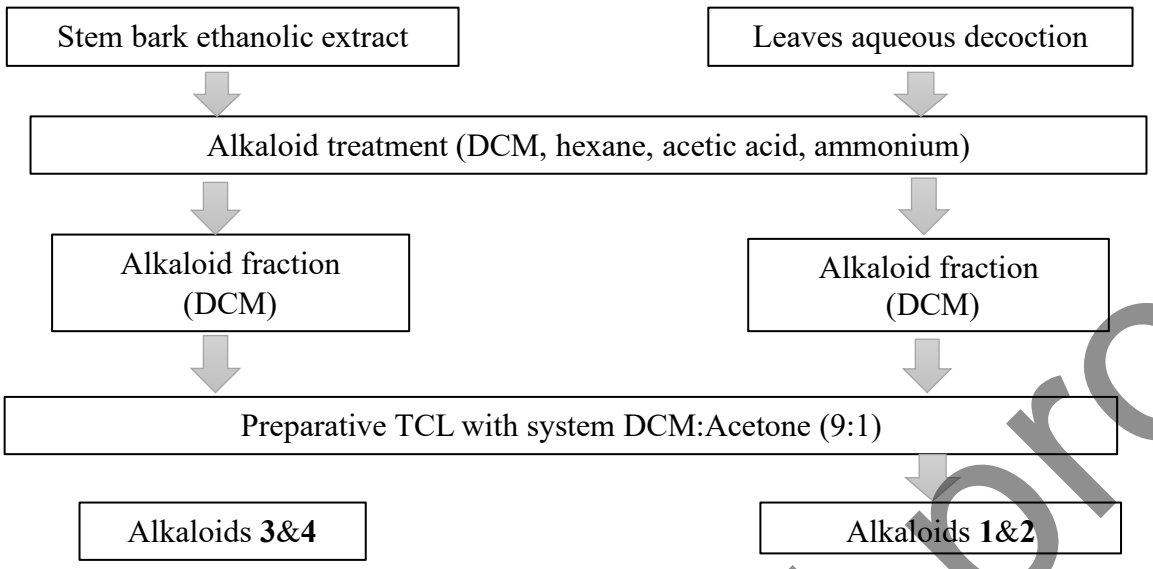
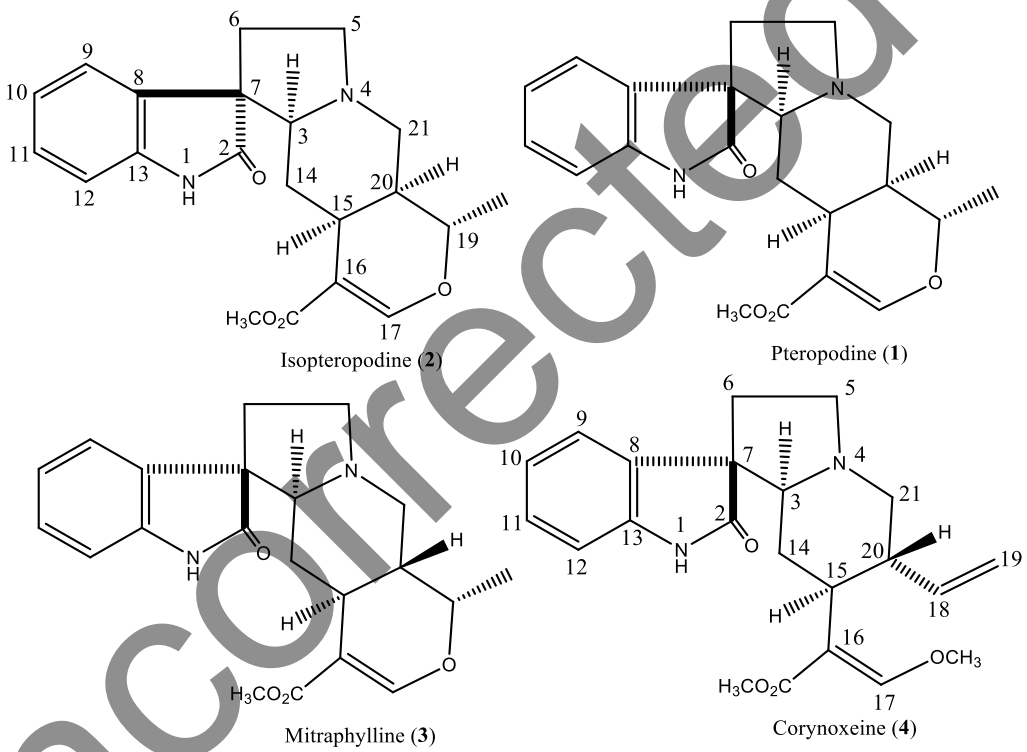


Figure 4: Flowchart of the whole study



Figures 5: Structures of pteropodine (**1**), isopteropodine (**2**), mitraphylline (**3**) and corynoxeine (**4**)

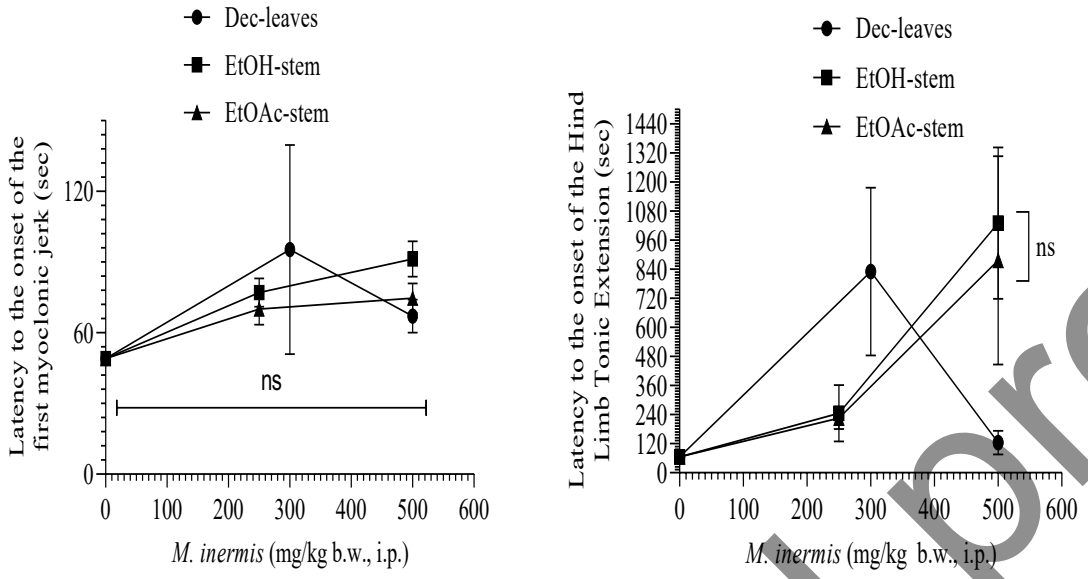


Figure 6: Anti-PTZ-induced seizures of some most active fractions: Onsets of first myoclonic jerk and Hind Limb Tonic Extension are expressed as mean±SD, n=7. Dec= decoction, EtOH= ethanol, EtOAc= ethyl acetate. The dose 0 mg/kg b.w. is control (group treated with distilled water). No significant difference between first myoclonic jerk onset of the control and treated groups. Compared to the control group, stem bark ethyl acetate and ethanol extract and leave decoction delayed noticeably HLTE occur ($p \leq 0.05$). Statistical analyses were carried out by one-way ANOVA with Dunnett's multiple comparison tests. Statistical significance levels: *$p \leq 0.05$; ns: non-significant difference; duration in seconds.

Table I: The screening of the different parts extract and fraction of *Mitragyna inermis* on PTZ-induced seizures in mice

Parts	**Extracts/fractions**	**Doses (mg/kg)**	Delay in HLTE occur
Leaves	Decoction	500	++
		300	+++
		150	+
	Ethyl acetate + Butanol + Acetone	500	++
		250	+
Stem bark	Ethanol	500	++++
		250	+++
	Ethyl acetate + Acetone	500	+++
		250	++
	Ethyl acetate	500	+++
		250	+
	Acetone	500	-
		250	-
Roots	Ethyl acetate + Acetone	500	-
		300	-

Noticeable delay of HLTE was recorded by comparison with the group treated with distilled water as a control; **n=3**: More than 10 min= ++++; 5 to 10 min= +++; 2 to 5 min= ++; less than 2 min= +, no activity=-