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Mechanisms of Anticonvulsant Action of Residual Aqueous Fraction (RAF) of the Ethanol Root Bark Extract of *Carissa edulis*

Jamilu Ya'u[1*], Abdullahi Hamza Yaro[2], Aliyu Muhammad Musa[3], Ahmed Abubakar[4], Sani Mohammed Yahaya[3], Ben Ahmadu Chindo[1,5], Joseph Akpoju Anuka[1], Isa Marte Hussaini[1,6]

1. Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria
2. Department of Pharmacology, Bayero University Kano, Kano, Nigeria
3. Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria
4. Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria
5. Department of Pharmacology and Therapeutics, Kaduna State University, Kaduna, Nigeria
6. Department of Pharmacology, University of Maiduguri, Maiduguri, Nigeria

[*] E-mail of the Corresponding author: yjamilu@abu.edu.ng; jameelyau@yahoo.com

Abstract

Preparations of *Carissa edulis* (Vahl) have been used in the Nigerian traditional medicine for the management of fever, sickle cell disease, epilepsy, pain cancer and inflammatory conditions for many years and their efficacy is widely acclaimed among the Hausa communities of Northern Nigeria. The possible mechanism(s) involved in the anticonvulsant action of residual aqueous fraction of ethanol root bark extract of *Carissa edulis* (RAF) were determined using flumazenil, naloxone, cyproheptadine, bisindolylmaleimide (BIM), ibuprofen and misoprostol. Flumazenil antagonized the anticonvulsant effect of both RAF and diazepam, while BIM promoted their anticonvulsant effect. However, naloxone and cyproheptadine did not affect the RAF anticonvulsant effect. Ketoprofen increased the RAF and valproate anticonvulsant activity while misoprostol did not alter their effects. The interaction studies revealed that GABAergic, glycine, serotonergic and opioid neurotransmission were found to be involved in the RAF anticonvulsant effects.

Keywords: Anticonvulsant, Bisindolylmaleimide, Flumazenil, GABA, serotonergic, opioids

1. Introduction

Plants extracts can be an important source of natural and safer drugs for the treatment of epilepsy (Meldrum, 1997). Extracts, fractions and pure compounds from several medicinal plants have been used in traditional medicinal treatment of epilepsy and have demonstrated anticonvulsant properties that need to be further investigated (Raza *et al.,* 1999). The World Health Organization (WHO) estimated that about 80% of the people that are living in developing countries use exclusively traditional medicines to treat their health problems (Eloff, 1998). Consequently, WHO recommended the initiation of programs designed to use medicinal plants more effectively in traditional health care systems (WHO/PRO, 1998). In developed countries more recently, interest has risen in the value of plants as sources of new drug candidates and in herbal medicines for healthy lifestyles (Aniagu *et al.,* 2004; Chindo, 1999).

Carissa edulis Vahl (Family: Apocynaceae) is a common medicinal plant found in the Northern part of Nigeria. Decoctions of *C. edulis* are used in folkmedicine for the treatment of a variety of diseases including, sickle cell anemia, toothache, ulcer, worm infestation, epilepsy, pain and inflammation (Ibrahim *et al.,* 2007) and their efficacy is widely acclaimed among the Hausa communities of Northern Nigeria. Previous studies suggested that extracts of *C. edulis* may contain biologically active principles with potential diuretic (Nedi *et al.,* 2004), analgesic (Ibrahim *et al.,* 2007), antimicrobial (Ibrahim *et al.,* 2005), antidiabetic (El-Fiky *et al.,* 1996), sedative, anticonvulsant and anxiolytic properties (Ya'u *et al.,* 2007; 2008; 2010).

2. Materials and methods

2.1 Plant Preparation

The root bark of *C. edulis* (Vahl) was collected in July 2010 at Basawa Village, Zaria, Nigeria. The plant sample was identified and authenticated by Taxonomist, Malam Umar Gallah in the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria and was given a voucher specimen number 601 for future reference.

The root bark samples were air dried at room temperature under shade until constant weight was obtained. The dried samples were size reduced. The powdered sample (100 g) was extracted with 700 ml of 70% $^{v}/_{v}$ ethanol in water using cold maceration method 14 days with occasional shaking. The filtrate was concentrated to dryness producing brown mass with pleasant smell.

2.2 Animals

Male Swiss Abino mice were obtained from Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were maintained at room temperature 25.0 ± 2.0 ℃, 12 h light and dark cycle, fed with standard animal feed (Feeds Masters, Ilorin, Nigeria) and water was provided ad libitum. The animals were used in compliance with the National Institute of Health Guide for the Care and use of Laboratory Animals (Publication No. 85 – 23,

revised 1985). The institutional approval number for the protocol was given as DAC/IW-OT/003-10.

2.3 Methodology

2.3.1 Effects of flumazenil on anticonvulsant activity of RAF of ethanol extract of Carissa edulis root bark and diazepam on PTZ-induced seizures in mice

The effects of selective GABAA-BZD receptor antagonist; flumazenil were also studied (File *et al.,* 1982; File and Pellow, 1985) on the anticonvulsant activity of the RAF in order to investigate the probable involvement of GABAA-BZD receptors. Two groups of 6 mice each were selected. In the first group, mice were given flumazenil (10 mg/kg) 15 min before the administration of *C. edulis* (5 mg/kg) extracts (45 min before the injection of PTZ). In the second group, the animals received flumazenil 15 min before the administration of diazepam (0.5 mg/kg) (45 min before the injection of PTZ). The anticonvulsant activity of the extract and diazepam in mice pretreated with flumazenil was assessed and compared with controls and extract treated animals.

2.3.2 Effect of bisindolylmaleimide III (BIM), naloxone and cyproheptadine on anticonvulsant activity of residual aqueous fraction (RAF) of ethanol root bark extract of Carissa edulis and valproate on pentylenetetrazole (PTZ)-induced seizures in mice

The effects of Protein kinase C (PKC) inhibitor, opioid antagonist (naloxone) and non-selective histamine and serotonin antagonist (cyproheptadine) on anticonvulsant activity of RAF were studied to investigate the probable involvement of PKC, serotonin, histaminergic and opioid pathways. The mice were divided into nine groups of six mice each. The first group received distilled water 10 ml/kg. The second and third groups were given RAF 600 mg/kg and valproate 200 mg/kg respectively. Fourth, fifth and sixth groups were pretreated with BIM (1 mg/kg), naloxone (0.3 mg/kg) and cyproheptadine (4 mg/kg) for 15 min followed by RAF (600 mg/kg) after 30 min PTZ (85 mg/kg) was administered subcutaneously. The same protocol was used for groups seventh, eighth and ninth for valproate. The anticonvulsant effects of RAF and valproate were noted and recorded.

2.3.3 Effects of ketoprofen and misoprostol on anticonvulsant activity of residual aqueous fraction (RAF) of ethanol root bark extract of Carissa edulis and diazepam (DZP) on pentylenetetrazole (PTZ)-induced seizures in mice

The effect of cyclooxygenase (COX) inhibitor (Ketoprofen) and prostaglandin analogue (misoprostol) on anticonvulsant activity of RAF was studied to investigate probable involvement of prostanoids pathways. The mice were divided into eleven groups containing six mice per group (n= 6). The first group that served as a control received PTZ (85 mg/kg). The second, third, fourth, fifth and sixth groups received RAF (600 mg/kg), ketoprofen (5 mg/kg), ketoprofen (10 mg/kg), Diazepam (0.5 mg/kg) and Misoprostol (200 µg/kg) respectively, followed by administration of PTZ (85 mg/kg) after 30 minutes of injection of the drugs. The interactive studies were performed on groups seventh, eighth, ninth, tenth and eleventh, which received treatment with the following drugs after pretreatment with ketoprofen (10 mg/kg) and misoprostol (200 µg/kg) for 15 min prior to the administration of RAF (600 mg/kg) and diazepam (0.5 mg/kg) followed by PTZ after 30 minutes for each group.

3. Results

3.1 Effect flumazenil on anticonvulsant activity of RAF of ethanol root bark extract of Carissa edulis on PTZ-induced seizures in mice

Flumazenil reversed the protection offered against PTZ-induced seizures by RAF and diazepam. It also significantly reduced the mean onset of seizures and mean onset of mortality (Table 1).

Table 1. Effect flumazenil on anticonvulsant activity of RAF of ethanol root bark extract of *Carissa edulis* on PTZ-induced seizures in mice

Treatment (mg/kg)	Mean onset of seizure (min)	% Seizure protection	Mean latency of mortality (min)	% Mortality protection
Control	4.67 ±0.42	0.0	17.0 ±3.18	16.7
RAF 600	12.67 ±0.67	50.0	19.33 ±4.33	50.0
DZP 0.5	17.0 ±0.0	83.3	17.0 ±0.0	83.3
RAF 600 + FLU 10	8.83 ±2.94	0.0	15.33 ±4.84	50
DZP 0.5 + FLU 10	12.33 ±5.36	50.0	0.0	100

Control: Distilled Water, FLU: Flumazenil was administered 15 min before RAF: Residual aqueous fraction, DZP: diazepam was administered 30 min intraperitoneally before the injection of PTZ (85 mg/kg) subcutaneously; Values are the Mean ± SEM, n = 6.

3.2 Effect of bisindolylmaleimide III (BIM), naloxone and cyproheptadine on anticonvulsant activity of residual aqueous fraction (RAF) of ethanol root bark extract of Carissa edulis on pentylenetetrazole (PTZ)-induced seizures in mice

Bisindolylmaleimide III (BIM), naloxone and cyproheptadine potentiated the protection offered against PTZ-induced seizures by RAF. However, they did not significantly delay both the mean onset of myoclonic seizures and mean onset of tonic hind limb extension (THLE)/mortality. These agents, naloxone and cyproheptadine reversed the anticonvulsant effect of valproate but BIM potentiated the protection of valproate against PTZ-induced seizures. All the agents showed no significant effect on the mean onset of myoclonic seizures and THLE/mortality (Table 2).

Table 2. Effect of naloxone, cyproheptadine and bisindolylmaleide (BIM) on residual aqueous fraction (RAF) of ethanol root bark extract of *Carissa edulis* on pentylenetetrazole-induced seizures in mice

Treatment (mg/kg)	Mean onset of myoclonic seizure (min)	Quantal protection	Mean onset of THLE/mortality
Control	6.20 ±1.02	$^{0}/_{6}$	12.00 ±0.71
RAF 600	5.50 ±0.96	$^{3}/_{6}$	10.17 ±1.42
RAF 600 + BIM 1	8.87 ±1.76*	$^{5}/_{6}$	13.00 ±2.12
RAF 600 + NAL 0.3	7.80 ±1.31	$^{5}/_{6}$	16.50 ±2.96*
RAF 600 + CYP 4	11.17 ±2.54*	$^{4}/_{6}$	14.20 ±2.08*
VAL 200	13.20 ±1.56	$^{5}/_{6}$	19.00 ±0.00*
VAL 200 + BIM 1	12.75 ±2.72	$^{6}/_{6}$	-
VAL 200 + NAL 0.3	14.40 ±3.64	$^{2}/_{6}$	18.00 ±1.35
VAL 200 + CYP 4	10.00 ±2.00*	$^{4}/_{6}$	10.00 ±2.00
ONE WAY ANOVA	F=2.336 α<0.05		Not sig

Data presented as Mean ± SEM, n=6; Control = Distilled Water; RAF = Residual Aqueous Fraction; BIM = Bisindolylmaleimide III; CYP = Cyproheptadine; NAL = Naloxone; VAL = Valproate ; *p<0.05 Student t-test

3.3 Effect of ketoprofen and misoprostol on anticonvulsant activity of residual aqueous fraction (RAF) of ethanol root bark extract of Carissa edulis on pentylenetetrazole (PTZ)-induced seizures in mice

The ketoprofen potentiated the protection offered against PTZ-induced seizures by RAF from 33.33% to 50% and diazepam from 66.67% to 100% respectively. However, Misoprostol had no effect on the anticonvulsant activity of the RAF. However, they did not significantly delay both the mean onset of myoclonic seizures and mean onset of tonic hind limb extension (THLE)/mortality. Ketoprofen at 10 mg/kg protected the mice against PTZ-induced seizures by 33.33% (Table 3).

4. Discussion

In an effort to determine whether $GABA_A$-BZD receptor complex is involved in RAF mediated anticonvulsant effects, flumazenil a non-specific antagonist of the benzodiazepine site in the $GABA_A$-BZD receptor complex was used (Brogden *et al.,* 1988). The ability of flumazenil to reverse the effect of RAF on the PTZ-induced seizures suggests that the anticonvulsant activity of RAF might be mediated via BZD site of the $GABA_A$-BZD receptors.

Naloxone, cyproheptadine and Bisindolylmaleimide III (BIM) were used respectively to investigate the involvement of opioid, histaminergic and serotonergic receptor pathways respectively in the anticonvulsant effects of RAF. RAF and valproate showed significant delay in the onset of myoclonic seizure, tonic hind limb extension and mortality induced by PTZ in the group pretreated with BIM. The fraction demonstrated a significant protection against mortality when concurrently administered with BIM. Thus, in both the valproate and the RAF groups pretreated with BIM there was augmentation of anticonvulsant activity against PTZ-induced seizures. Though it was expected that BIM would have an antagonistic effect against the anticonvulsant agents since it is a potent $5HT_3$ antagonist that blocks the serotonergic pathways (Steven *et al.,* 1999), thus decreasing the activity of serotonin in the brain and consequently decreasing seizure threshold, leading to increase in convulsion. However, the fraction (RAF) and valproate respectively inhibited the effect of BIM thus making RAF and valproate potent anticonvulsant against $5HT_3$ antagonist (BIM); this indicates that their anticonvulsant activity may be partly via the serotonergic pathway.

Table 3. Effect of ketoprofen and misoprostol on residual aqueous fraction (RAF) of ethanol root bark extract of *Carissa edulis* on pentylenetetrazole-induced seizures in mice

Treatment (mg/kg)	Mean onset of myoclonic seizure (min)	Quantal protection	Mean onset of THLE/mortality
Control	7.33 ±1.15	$^{0}/_{6}$	12.83 ±2.06
RAF 600	7.73 ±1.51	$^{2}/_{6}$	14.25 ±2.17
RAF 600 + KET 10	10.00 ±1.29	$^{3}/_{6}$	9.67 ±1.33
RAF 600 + MIS 200	10.75 ±3.15	$^{2}/_{6}$	9.00 ±1.00
DZP 0.5	3.17 ±1.90	$^{4}/_{6}$	-
DZP 0.5 +KET 10	-	$^{6}/_{6}$	-
DZP 0.5 + MIS 200	-	$^{6}/_{6}$	-
KET 5	7.17 ±0.89	$^{0}/_{6}$	12.33 ±1.52
KET 10	6.67 ±1.50	$^{2}/_{6}$	10.00 ±1.35
ONE WAY ANOVA	F=0.957 α<0.478		F=2.423 α<0.048

Data presented as Mean ±SEM, n=6, Control = distilled water; RAF = Residual Aqueous Fraction; DZP = Diazepam; KET = Ketoprofen; MIS= Misoprostol

However, studies have shown that at downstream signaling pathways, GABA receptors are basally phosphorylated on a serine residue by a Protein Kinase C (PKC) dependent pathway (Nicholas *et al.*, 2012). PKC inhibitors such as BIM III abolish basal phosphorylation, increasing receptor activity, thus BIM increase GABA receptor activity, thereby increasing GABAergic transmissions, potentiating the activity of anticonvulsant agents such as RAF and valproate that act via GABAergic pathways. This may be the probable mechanism by which BIM potentiates the anticonvulsant effect of both RAF and valproate respectively.

RAF significantly protected mice against PTZ-induced seizure in the naloxone pretreated animals. There was a significant delay in the onset of myoclonus seizure, tonic hind limb extension and mortality in mice. Naloxone may induce or potentiate seizure by blocking opioid receptors and/or antagonizing GABA neurotransimission mediated by $GABA_A$ receptor channel complex. Several lines of behavioural and neurochemical evidence indicate $GABA_A$ antagonistic properties of naloxone (Svensson, 2000). Therefore, the ability of RAF to protect against naloxone-induced seizure suggests the involvement of the GABAergic and/or opioid receptors pathway, in the anticonvulsant effects of RAF.

The anticonvulsant effects of valproate against PTZ-induced seizure was abolished in the naloxone pretreated animals. Despite valproate increases GABA concentration by inhibiting GABA transaminase and stimulating glutamate decarboxylase (Hariton *et al.*,1984), in this study, sodium valproate could not alleviate PTZ-induced seizures in naloxone-pretreated mice even at relatively high doses suggesting that the anticonvulsant activity of valproate might not involve opioid receptor pathways.

The effect of RAF significantly delayed the PTZ-induced myoclonus seizure, tonic hind limb extension and mortality in mice pretreated with cyproheptadine. RAF also protected the mice pretreated with cyproheptadine against PTZ-induced tonic hind limb extension and death. Studies have shown that decrease in the level of serotonergic and histaminergic neurotransmissions in the brain reduces seizure threshold (Singh and Goel, 2010). These results therefore, suggest that RAF inhibited the proconvulsant effects of cyproheptadine. Thus, implicating the serotonergic and histaminergic neurotransmission systems in the anticonvulsant effects of the fraction given that cyproheptadine is an established antagonist of H_1, $5HT_2A$, $5HT_2B$, $5HT_2C$

and $5HT_1$ receptors (Peroukta, 1988).

Furthermore, the protective effects of RAF against PTZ-induced seizures in mice pretreated with cyproheptadine suggest modulatory effects on central GABAergic inhibitory neurotransmission (Stanley, 1995). It is probable that RAF promotes the activation of GABA receptors thereby potentiating GABAergic inhibition and/or histaminergic and serotonergic pathways. Antagonism of this process by cyproheptadine would have led to a significant decrease in GABA level resulting in suppression of anticonvulsant effect of the fraction, however, RAF showed significant protection against proconvulsant effect of cyproheptadine.

In the group pretreated with cyproheptadine, followed by valproate results showed a significant ($p<0.05$) increase in the time of onset of myoclonic seizure and tonic-clonic hind limb extension and mortality with decreased protection against death. It is an indication of the fact that cyproheptadine reduces seizure threshold, increasing severity of seizure probably due to its action via the serotonergic pathway, antagonizing 5HT receptors and decreasing serotonin levels in the brain (Singh and Goel, 2010). Valproate on the other hand acts via the GABAergic pathway, increasing GABA concentration, thereby potentiating GABAergic inhibition (Stanley, 1995). The decrease in the anticonvulsant effect of valproate in the presence of cyproheptadine is also an indication of the fact that valproate anticonvulsant activity could be in part via the serotonergic pathway.

Ketoprofen (NSAIDs) and misoprostol were employed to determine the involvement of prostanoids in the anticonvulsant activity of RAF. The results obtained revealed that NSAIDs (ketoprofen) significantly increase the level of protection against PTZ-induced seizures. Similarly, previous studies demonstrated the protective effects of NSAIDs in various models of epilepsy (Dhir *et al.,* 2008).

This indicated the protective effect of NSAIDs in PTZ- induced seizure in mice and suggests that RAF could mediate its effect via blockade of COX enzymes (unpublished data showed analgesic and anti-inflammatory effect of RAF).
Different classes of NSAIDs have been shown to decrease the threshold of PTZ induced seizure in mice (Mirhadi, 2011). However, the actual role of NSAIDS or prostaglandins on seizure may depend on the type of seizure and part of the brain affected. The role of COX isoforms and prostaglandins in brain diseases have been extensively reviewed by Minghetti and Pocchiari (2007), yet the exact mechanisms by which prostaglandins affect seizure remain unclear. However, evidences suggest the involvement of Gama amino butyric acid (GABA). Prostaglandins have been shown to contribute to epilepsy through modulation of adrenergic and glutamatergic neurotransmission and regulation of excitability of the membranes (Minghetti and Pocchiari, 2007).

5. Conclusion

In trying to establish the mechanism of RAF anticonvulsant activities. The interaction study with flumazenil, naloxone, bisindolylmaleimide, cyproheptadine, ketoprofen and misoprostol suggests that the actions of RAF may be via serotonergic, histaminergic and opioid neurotransmission pathways, in addition to the GABA-BDZ receptor channel complex.

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