

Antisnake venom activity and isolation of quercetin from the leaf of *Neocarya macrophylla* (Sabine) Prance ex F. White (Malpighiales: Chrysobalanaceae)

A. J. Yusuf^{1,*}, M. I. Abdullahi¹, A. M. Musa², A. K. Haruna², V. Mzozoyana³ and H. Abubakar⁴

¹Department of Pharmaceutical and Medicinal Chemistry. Usmanu Danfodiyo University. Sokoto. Nigeria. *Email: amynajega@gmail.com.

²Department of Pharmaceutical and Medicinal Chemistry. Ahmadu Bello University. Zaria. Nigeria.

³School of Chemistry and Physics. University of KwaZulu-Natal. Westville. Durban. South Africa.

⁴Department of Chemistry. Sokoto State University. Sokoto. Nigeria.

Abstract. Snake envenomation is a major cause of death and disability in many developing countries. *Neocarya macrophylla* (Sabine) Prance ex F. White (Malpighiales: Chrysobalanaceae) have been reportedly used in traditional medicine to treat snake envenomation. Bioassay-guided isolation of antivenom principles was carried out on the leaf of *N. macrophylla* against *Naja nigricollis* venom. The methanol extract of *N. macrophylla* leaf and its ethylacetate and *n*-butanol fraction significantly ($P < 0.05$) protected mice against venom-induced lethality with 100% survival rate and there was remarkable inhibition of the poisonous effects of PLA₂ enzyme by the extracts and the fractions. Encouraged by this result, the ethylacetate soluble fraction was subjected to purification using vacuum liquid chromatography and gel filtration which led to the isolation of quercetin as the bioactive principle. The identity of the compound was determined on the basis of chemical tests, and by comparison of its ¹H-NMR data with literature, this is the first report of isolation of this compound from the leaf of the plant. However, the results of the study suggests that the leaf of *N. macrophylla* possess significant antisnake venom activity which provide the scientific basis for its use in traditional treatment of snakebites.

Keywords: *Neocarya macrophylla*; Leaf; Antivenom; Quercetin; NMR.

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ORCID

0000-0002-4557-859X
A. J. Yusuf

0000-0002-9049-9057
M. I. Abdullahi

0000-0001-7985-9157
A. M. Musa

0000-0003-1164-287X
A. K. Haruna
0000-0002-6679-1541
V. Mzozoyana
0000-0003-1039-5782
H. Abubakar

Introduction

Snakebites is a neglected public health concern in many developing countries (Alirol et al., 2010; Chippaux, 2011) especially in the rural areas where access to healthcare facilities is severely limited (Aleku et al., 2014; Dechambenoit, 2016). Consequently, many snakebite patients rely on alternative herbal medicinal antivenom products with no or limited scientific evidence of safety and efficacy.

Ginger bread plum *Neocarya macrophylla* (Sabine) Prance ex F. White (Malpighiales: Chrysobalanaceae) has been used folklorically in Northern Nigeria to treat several diseases, including infectious diseases and snake bites (Yusuf et al., 2015a). Previous phytochemical investigations of other members of the Chrysobalanaceae Family have been adequately documented. Chemotaxonomically, flavonoids, steroids and terpenes are the major constituents of the plant family (Carnevale Neto et al., 2013). The ease of availability of this plant in Nigeria and elsewhere prompted us to conduct antsnake venom activity and phytochemical analysis of the stem bark of *N. macrophylla* in which stigmaterol and bis-(5,7-diacetyl-catechin-4'- α -rhamnopyranoside) were isolated (Yusuf et al., 2015a; Yusuf et al., 2015b). Indeed, we have recently demonstrated the antimicrobial activity of stigmaterol isolated from *N. macrophylla*, exhibiting broad spectrum antimicrobial activity thereby validating its use for the treatment of infectious diseases (Yusuf et al., 2018).

The correlation of the anecdotal use and observed biological activity of

the stem-bark of *N. macrophylla* strengthen us to provide scientific basis for the use of the leaf of the plant in the treatment of snakebites. We report herein, the antsnake venom activity of the methanol leaf extract of *N. macrophylla* and against *Naja nigricollis* venom and isolation of quercetin, a known flavonoid. Fractionation of the MeOH leaf extract and the subsequent purification of a promising bioactive fraction (EtOAc soluble fraction) led to the isolation and structure elucidation of a bioactive principle, quercetin.

Methodology

NMR data were recorded on a Bruker AVANCE spectrometer (600 MHz) with residual solvent as internal standard. Melting point was determined on an Electro thermal melting point apparatus. Thin layer chromatography (TLC) was carried out using silica gel 60 GF₂₅₄ pre-coated aluminium sheets by Sigma Aldrich, Germany. Vacuum liquid chromatography was conducted using LOBA Cheme silica gel (60-200) mesh in a sintered glass funnel while gel filtration chromatography was performed using sephadex LH-20 (Sima, Spruce Street, St. Louis, MO, USA). Spots on TLC plates were visualized by spraying with 10% H₂SO₄ followed by heating at 105 °C for 10 min.

Plant sample

The plant sample of *N. macrophylla* was collected in October, 2015 at Jega Local Government Area of Kebbi State. It was identified by Namadi Sanusi at the Herbarium Unit, Department of Biological Sciences, Ahmadu Bello University by comparing

with herbarium reference voucher specimen (No. 3197). The leaf was shade dried, pulverized to powder, labelled and stored at room temperature for use.

Experimental animals

Swiss albino mice of either sex weighing (18-24 g) were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were fed with laboratory diet and water *ad libitum* and maintained under standard conditions (12 h light and 12 h dark cycle) in propylene cages at room temperature. All experimental procedures were approved by the Animal Right and Ethics Committee of the University.

Snake venom

The venom of an adult *N. nigricollis* was collected by the milking method of Macfarlane (1967). It was pooled, lyophilized and stored at 4 °C until required. The venom was subsequently referred to as the crude venom of *N. nigricollis*.

Antisnake venom

Lyophilized polyvalent snake venom antiserum sourced from VINS Nioproduct Limited, Andhra Pradesh, India (Batch. number: 01AS14054, Manufacturing Date: July 2015, Expiry date: June 2018) was used for the study.

Preparation of plant material

The leaf of *N. macrophylla* (1.9 kg) was exhaustively extracted with 90 % MeOH and the combined extracts were evaporated *in vacuo* to dryness to afford MeOH leaf extract (288 g). Some part (200 g) of the extract was suspended in distilled water and successively partitioned into *n*-hexane, chloroform, EtOAc and *n*-butanol fractions.

LD₅₀ determination

The intraperitoneal LD₅₀ of the MeOH leaf extract and its fractions (EtOAc and *n*-butanol) was determined

according to the method described by Lorke (1983).

Lethality assay of the venom

The LD₉₉ of the venom was determined by the method described by Theakston and Reid (1983) and the LD₉₉ value was calculated by probit analysis (Finney, 1977) of death occurring within 24 h of venom injection.

***In vivo* detoxifying effect**

The method described by Theakston and Reid (1983) was employed. Twenty mice (20) were divided into five groups (n = 4). Group 1 (control) received 1 mL/kg of saline. Groups 2, 3 and 4 (test groups) received 20, 40 and 80 mg/kg of the MeOH leaf extract, respectively. The groups were then injected with MLD (LD₉₉, 5.75 mg/kg) of the reconstituted venom 30 min after injecting the extract. The route of administration was intraperitoneal (*i.p.*). All animals were observed for mortality for 24 h. The experiment was repeated for the EtOAc (20, 40 and 80 mg/kg) and *n*-butanol 5, 10 and 20 mg/kg fractions.

***In vitro* detoxifying effect**

The method described by Abubakar et al. (2000) was employed in this study. Twenty (20) mice were divided into four groups (n=5). Group 1 (control) received the MLD (LD₉₉, 5.75 mg/kg) of *N. nigricollis* venom only. Groups 2, 3 and 4 (treatment groups) received an equivalent of the MLD of the venom which was previously incubated at 37 °C for 10 min with 20, 40 and 80 mg/kg of the MeOH leaf extract and the animals were observed for mortality for 24 h. The route of administration was intraperitoneal. The experiment was repeated for the EtOAc (20, 40 and 80 mg/kg) and *n*-butanol 5, 10 and 20 mg/kg fractions.

Phospholipase A₂ (PLA₂) assay

The method of Tan and Tan (1988) was used to conduct the study.

Equal volumes of substrate comprising calcium chloride (18 mM), sodium deoxycholate (8.1 mM) and egg yolk were mixed and stirred for 10 min to get homogenous egg yolk suspension. The pH of the suspension was adjusted to 8.0 by addition of sodium hydroxide (1 M). Snake venom (0.3 mg/mL) was added to the above mixture (15 mL) to initiate the process of hydrolysis and saline was used as control. Decrease in pH of the suspension was noted after two minutes with the help of a pH meter. A decline in 1.0 pH unit corresponds to 133 μ moles of fatty acid released in the egg yolk mixture. The enzymatic activity of phospholipase A₂ was calculated from the data obtained as micromoles of fatty acid released per minute (Tan and Tan, 1988).

To test the antsnake venom potential of *N. macrophylla*, snake venom (0.1 mg) was pre-incubated with the MeOH extract of the plant and its fractions (0.5 mg/mL) to neutralize the hydrolytic action of PLA₂. Protection offered by the MeOH leaf extract, EtOAc and *n*-butanol fractions against the phospholipases.

Isolation of compound

The EtOAc fraction was chromatographed on silica gel vacuum liquid chromatography using *n*-hexane:EtOAc mixtures as solvent system. Thirty (30 mL) each of a total of 80 fractions were collected and merged based on their TLC profile to afford seven (7) major fractions E1 – E7. Further purification of fraction E2 using sephadex LH-20 afforded a yellow solid substance (Compound **1**). It was subjected to chemical test and spectroscopic analysis to elucidate its chemical structure.

Spectral data

Quercetin (7 mg). Yellow solid substance; m.p 310-312 °C. ¹H-NMR (CD₃OD, 600 MHz): δ_H 7.76 (1H, d, $J=2.2$ Hz, H-2'), 6.92 (1H, d, $J=8.4$ Hz, H-5'), 7.67 (1H, dd, $J=2.2, 8.4$ Hz, H-6'), 6.42

(1H, d, $J=2.2$ Hz, H-6), 6.22 (1H, d, $J=2.2$ Hz, H-8).

Results and discussion

N. macrophylla has been used in ethnomedicine to treat snakebite victims (Yusuf et al., 2015a); this study was performed to establish any scientific evidences justifying its use for this purpose. First, acute toxicity profile of methanolic extract and the resulting fractions was investigated. The median lethal dose LD₅₀ of the MeOH leaf extract was found to be 288 mg/kg; values of 565 and 141 mg/kg were obtained for EtOAc fraction and *n*-butanol fractions indicating that the extract and its fractions are toxic (Lorke, 1983); Similar value was reported for the MeOH stem bark extract of the plant (Yusuf et al., 2015a). The MLD (LD₉₉) of the *N. nigricollis* venom estimated by probit analysis was 5.75 mg/kg. Although, Ode and Asuzu (2006) reported a value (6 mg/kg) for the same species of snake. However, higher values (9.55 mg/kg) and (9.7 mg/kg) were reported by Abubakar et al. (2000) and Isah et al. (2015) respectively. This variability observed in the minimum lethal doses of the venom might be due to the difference in venom composition which is attributed to geographic location, subspecies, season, age and diet of the snakes (Chippaux et al., 1991; Daltry et al., 1996).

In vivo antivenom activity was observed for the MeOH leaf extract. There was 20 % protection at 20 and 40 mg/kg; no protection was observed at the highest dose (80 mg/kg). However, activity was diminished after partitioning the extract into EtOAc and *n*-butanol fractions (Table 1). Interestingly, the MeOH leaf extract and its fractions (EtOAc and *n*-butanol) significantly ($P < 0.05$) protected mice against venom-induced actions *in vitro* with 100 % survival rate at the graded doses employed (Table 2). This was further validated by subjecting the extract and

fractions to phospholipase A₂ (PLA₂) enzyme acidimetric assay. In this assay, PLA₂ enzymes were hydrolyzed and free fatty acids were released in the presence of sodium deoxycholate. *N. nigricollis* venom was found to liberate free fatty acid which was measured in terms of decrease in pH of egg suspension. The MeOH leaf extract and its fractions were

able to inhibit the hydrolytic action of PLA₂ activity (Figure 1). The MeOH extract showed superior inhibitory effect when compared to its fractions, displaying 52.5 % protection from the poisonous effect of PLA₂; while the *n*-butanol and EtOAc fractions which displayed 33.3 and 31.5 % inhibition, respectively.

Table 1. *In vivo* detoxifying effect of extract/fraction of *N. macrophylla* against *N. nigricollis* venom.

Treatment component	Control group LD ₉₉	% Survival of mice within 24 hours on different treatment doses		
		T1	T2	T3
MEL Extract	0	20	20	0
EF Fraction	0	0	0	0
BF Fraction	0	0	0	0

Key: MEL = methanol leaf extract; EF = ethylacetate fraction; BF = *n*-butanol fraction; T = Treatment dose. T1 = 20 mg/kg; T2 = 40 mg/kg; T3 = 80 mg/kg. *P* < 0.05 compared with control were considered significant (Chi-Square test); (n = 5).

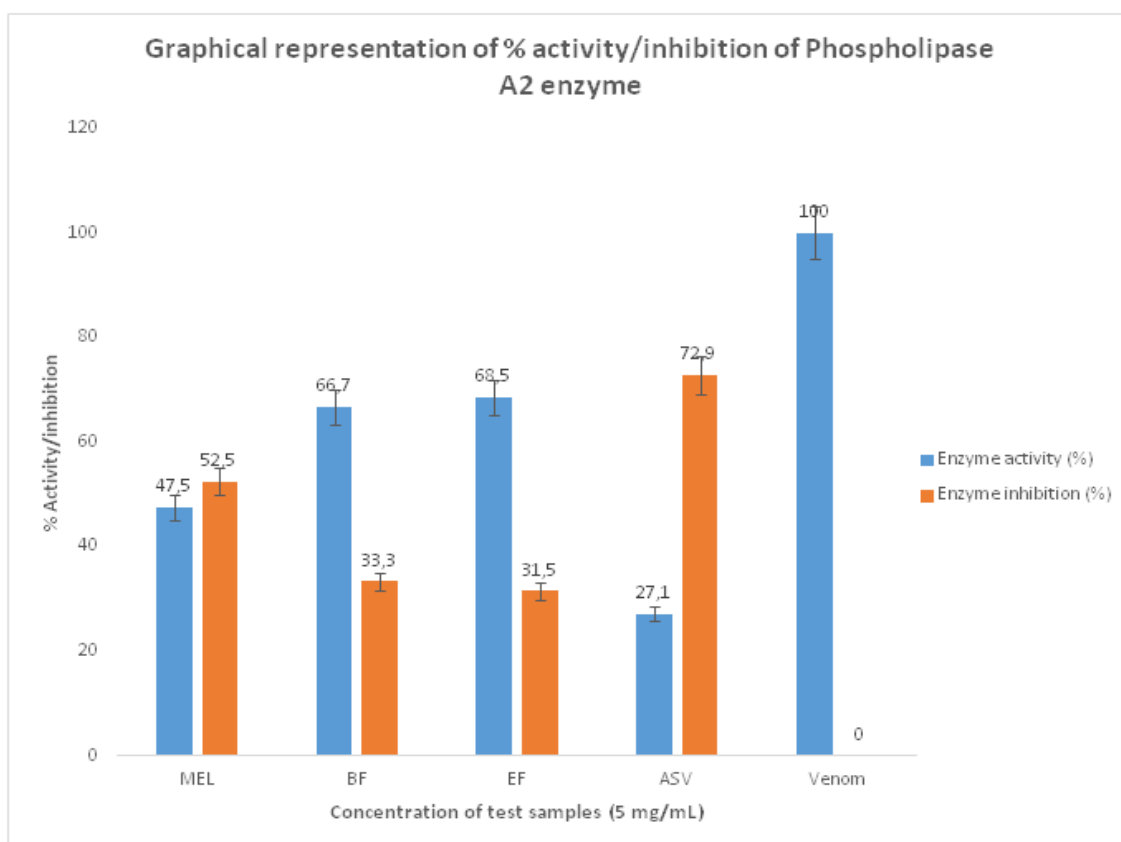


Figure 1. PLA₂ Enzyme activity and inhibition by the extract/fractions of *N. macrophylla*. MEL=methanol leaf extract; BF=*n*-butanol fraction; EF=ethylacetate fraction; ASV=antisnake venom.

Table 2. *In vitro* detoxifying effect of extract/fraction of *N. macrophylla* against *N. nigricollis* venom.

Treatment component		Control group LD ₉₉	% survival of mice within 24 h on different treatment doses		
			T1	T2	T3
MEL	Extract	0	100*	100*	100*
EF	Fraction	0	100*	100*	100*
BF	fraction	0	100*	100*	100*

MEL=methanol leaf extract; EF=ethylacetate fraction; BF=n-butanol fraction; T = Treatment dose. T1 (mg/kg) = MEL, EF (20); T2 (mg/kg) = MEL, EFL (40); T3 (mg/kg) = MEL, EF (80). T1 (mg/kg)= BF(5); T2 (mg/kg)= BF(10); T3 (mg/kg)= BF(20). $P < 0.05$ compared with control were considered significant (Chi-Square test); (n = 5).

Inspired by the antivenin activity of the MeOH extract, effort at isolating a bioactive constituent was explored. The EtOAc fraction of the MeOH leaf extract of *N. macrophylla* was subjected to chromatographic studies which led to the isolation of a known flavonoid. It reacted positively to Shinoda test suggesting the presence of phenolic nucleus (Silva et al., 1998). The ¹H-NMR spectra of the isolated compound exhibited chemical shift values typical of flavonoids (Mabry et al., 1970; Sani et al., 2015). The presence of 1, 2, 3, 5-tetrasubstituted benzene ring A was revealed *via* the proton shifts at δ_H 6.42 (1H, d, J = 2.2 Hz)

and δ_H 6.22 (1H, d, J = 2.2 Hz) representing H-6 and H-8 respectively. The proton resonances at δ_H 7.76 (1H, d, J = 2.2), δ_H 6.92 (1H, d, 8.4 Hz) and δ_H 7.67 (1H, dd, J = 2.2, 8.3 Hz) indicated the presence of a 1, 3, 4-trisubstituted aromatic ring B. The chemical shift for these protons correspond to the chemical shift values reported for Quercetin by Mabry et al. (1970) and Sani et al. (2015) (Table 3). Based on the above data and comparison with reported literature (Mabry et al., 1970), the compound was confirmed to be 3, 5, 7, 3', 4'-pentahydroxyflavonol or quercetin (Figure 2).

Table 3. ¹H-NMR data of compound 1 compared with reported literature.

Position	¹ H-NMR of compound 1 (J = Hz)	¹ H-NMR (Mabry et al., 1970)
2		
3		
4		
5		
6	6.22 (d, 2.2 Hz)	6.21 (d, 1.98 Hz)
7		
8	6.42 (d, 2.2 Hz)	6.42 (d, 1.98 Hz)
9		
10		
1'		
2'	7.76 (d, 2.2 Hz)	7.76 (d, 2.04 Hz)
3'		
4'		
5'	6.92 (d, 8.4 Hz)	6.91 (d, 8.34 Hz)
6'	7.67 (dd, 2.2, 8.4 Hz)	7.66 (dd, 2.1, 8.40 Hz)

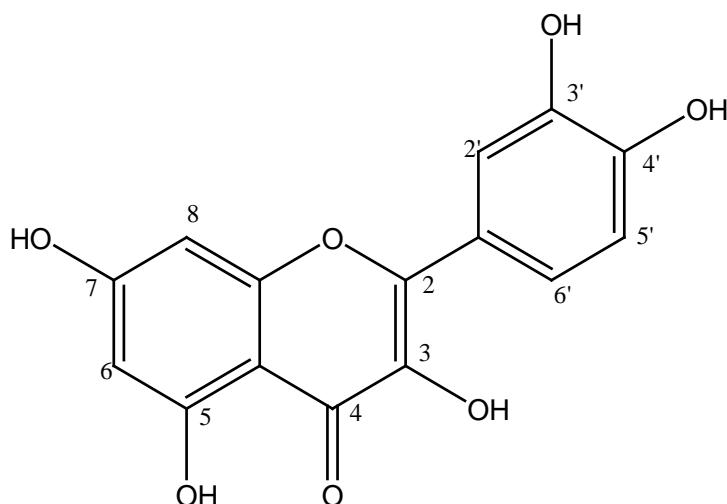


Figure 2. 3, 5, 7, 3', 4'-pentahydroxyl flavonol (quercetin).

The mechanism of action of the active constituents of plant extracts have been suggested to be associated with enzymes (Tsai et al., 1980; Bhat et al., 1991); toxic effects of snake bites are associated with enzymes (Stocker, 1990) mainly through the hydrolytic action of phospholipase A₂ (PLA₂) which is thought to be the most toxic component (Mahanta and Mukherji, 2001) implicated in tissue necrosis and haemorrhage (Melo and Ownby, 1999). The extract and fractions were observed to significantly ($P < 0.05$) protect the laboratory animals against venom-induced lethality *in vitro*. This effect might be linked to activity of the extract and fractions against the toxic enzymes (Haslam, 1989) suggesting the leaf of *N. macrophylla* might have prophylactic effect.

Most bioactive molecules isolated from medicinal plants with antiophidic activity are phenolic compounds with aromatic ring substituted by one or more hydroxyl groups (Nicholson and Hammerschmidt, 1992). However, studies have shown that hydroxyl group at position 5, double bond and double bonded oxygen in the oxane ring are important for the overall capacity of flavonoids to inhibit PLA₂ activity. In addition to that, hydroxyl groups in

positions 3' and 4' are required for the selective inhibition of PLA₂-II (Chippaux et al., 1997; Lindahl and Tagesson, 1997). The observed antivenin activity could be linked to the presence of quercetin.

In addition, quercetin is a plant derived polyphenolic compound and have been reported to possess different biological activities including antisnake venom (Haslem, 1989), cardiovascular protection, anticancer, anti-tumor, anti-inflammatory, antidiabetic, anti-ulcer, anti-viral, anti-allergy, antihypertensive, anti-infective, gastro protective and immunomodulatory effects (Alexander et al., 2016).

Conclusion

In summary, we have shown that methanolic leaf extract of *N. macrophylla* possessed antisnake venom activity. Fractionation and purification of the crude MeOH extract led to the isolation of a compound identified as quercetin. Although the antisnake venom activity of the isolated quercetin could not be evaluated due to paucity of the material, the flavonoid quercetin has previously been shown to exhibit different pharmacological activities including antisnake venom. Effort is ongoing in our laboratory to further isolate this

compound in sufficient quantity to allow for detailed bioactivity profiling of quercetin and to evaluate its potential as lead in the development novel antisnake venom therapeutic agent.

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Conflicts of interest

Authors declare that they have no conflict of interests.

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